PROCEEDINGS OF THE

SIXTH INTERNATIONAL SYMPOSIUM ON PERTUSSIS

Department of Health and Human Services
United States Public Health Service
Food and Drug Administration

International Sympos. on pertussis

PROCEEDINGS OF THE
SIXTH INTERNATIONAL SYMPOSIUM ON PERTUSSIS

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National Institutes of Health
Bethesda, Maryland
September 26–28, 1990

Department of Health and Human Services
United States Public Health Service
Food and Drug Administration

1990

Cite this publication as follows:

Manclark, C.R. (ed.) 1990. Proceedings of the Sixth International Symposium on Pertussis. Department of Health and Human Services, United States Public Health Service, Bethesda, Maryland. DHHS Publication No. (FDA) 90-1164. 408 pages

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Preface

As each of the international symposiums on pertussis have convened, it has become the custom to recount the time, place, and purposes of previous symposiums; that tradition will continue here.

In 1972, the Permanent Section of Microbiology Standardization of the International Association of Microbiological Societies organized the first **Symposium on Pertussis Immunization** in Prague. Seven years later, in 1979, the second **International Symposium on Pertussis** was held in Bilthoven. The **Third International Symposium on Pertussis** in Bethesda in 1978 was sponsored by the Bureau of Biologics, National Institute of Allergy and Infectious Diseases, Centers for Disease Control, International Association of Biological Standardization, and the Fogarty International Center of the National Institutes of Health. The **Fourth International Symposium on Pertussis** was held in 1984 at the World Health Organization in Geneva under the sponsorship of the International Association of Biological Standardization and the World Health Organization. In 1988, the **Fifth International Symposium on Pertussis** was held in Copenhagen and was sponsored by the Tokai University and the Tokai University European Center. The **Sixth International Symposium on Pertussis** was held in Bethesda in 1990 with sponsorship of the Center for Biologics Evaluation and Research. These symposiums have served to review, refocus, and redirect pertussis research, but there have been other meetings and workshops which have been important as well.

The 1975 seminar on **Pertussis Vaccine: Current Status**, held at the Annual Meeting of the American Society for Microbiology in New York City, was the first public exposition of a working hypothesis that we articulated in 1967. The hypothesis broke from the traditions of classical pertussology and proposed that basic research on physiology, genetics, and immunochemistry of *Bordetella pertussis*, and studies of the host parasite interactions in pertussis would be prerequisite for an improved pertussis vaccine and recognized the problems in evaluating the new vaccines and proving their clinical safety and efficacy. These topics continue to serve as the focus of our research and are the subjects of symposiums and other important meetings held since 1975.

In 1977 the **All-Union Symposium on Immunology and Epidemiology of Whooping Cough** was held in Minsk. At an **International Symposium on Bacterial Vaccines** convened in 1980 at the National Institutes of Health in Bethesda, among other things reported was the first purification to homogeneity of filamentous hemagglutinin and a method with commercial applications for the purification of filamentous hemagglutinin and pertussis toxin.

Issues related to the evaluation of candidate vaccines were examined in a workshop entitled **New Pertussis Vaccines — Laboratory and Clinical Evaluation** held in 1982 in Bethesda. This was followed by a **Workshop on Acellular Pertussis Vaccines** held in Bethesda in 1986 and another workshop on the **Status of Acellular Pertussis Vaccines and Swedish Trial Update** held in Bethesda in 1988. The **FEMS Symposium on Pertussis** was held in Berlin in 1988, and there is a continuum of pertussis meetings held in conjunction with conferences of a variety of scientific societies throughout the world. The level of research is intense, the quality of science is excellent, and every effort has been made to communicate new information and take innovative approaches to solve problems.

This is a very special time in understanding pertussis and pertussis vaccine. In less than twenty years, which is only a moment in the history of man's experience with pertussis, we have progressed from a rudimentary knowledge of pertussis and its etiologic agent to a point where it may be possible to obtain very high levels of control or eradication of the disease. Past generations did not have the knowledge to understand pertussis or develop the vaccine, and future generations may not appreciate today's problems with the disease or the vaccine. We all are privileged to have been a part of the interval when pertussis was understood and a definitive immunoprophylaxis was developed.

In the introduction to his book *Broca's Brain*: *Reflections on the Romance of Science*, Carl Sagan has written eloquently about the excitement of being associated with momentous events of science. I have paraphrased Dr. Sagan's words to reflect this moment in pertussis research.

> If we were scientists in 1990 . . . we could have wondered, pondered, and speculated about the cause, treatment, and prophylaxis for pertussis, but we could have done little or nothing about these issues. Had we been born fifty years from now, the issues would have been solved. Our children will have been taught the answers before most of them will have had an opportunity to formulate the questions. By far the most exciting, satisfying, and exhilarating time to be alive is the time in which we pass from ignorance to knowledge of fundamental issues; the age where we begin in wonder and end in understanding. In all of the four-million-year history of the human family, there is only one generation privileged to live through that unique transitional moment; that generation is ours.

The *Proceedings of the Sixth International Symposium on Pertussis* was published quickly in order to assist scientists in their work and public health administrators in their policy determinations. Manuscripts were submitted prior to the Symposium and reviewed with the authors during the meetings. In order to save time, however, the papers were published without returning the final edited page proofs to the authors for review. Accordingly, I accept the responsibility for errors incurred during editing and printing.

Charles R. Manclark
Bethesda, Maryland
October, 1990

Opening Address

CHARLES R. MANCLARK

Over the past 20 years there has been a rebirth of interest in pertussis research, and this was accompanied by a significant influx of scientists, ideas, and information to the field. Much of the renewed interest was reflected in the **Third International Symposium on Pertussis** held in this auditorium in 1978. Major contributions to the immunochemistry of *Bordetella pertussis* have been made since the 1978 meeting, and this information has served as the basis for determining the formulation of the first generation acellular vaccines destined to replace the whole cell pertussis vaccines.

The first generation acellular vaccines should be less reactogenic, and should permit larger amounts of specific immunogens to be administered in a dose with an expected immunity of longer duration. As most of you know, we are well on our way to evaluating the safety and efficacy of these first generation vaccines and such studies should provide us with an alternative to the whole cell vaccines in the very near future.

While much of the attention of physicians, parents, and the press has been directed at the prospect of a vaccine to replace the whole cell pertussis vaccines, probably the most significant recent research has dealt with issues relating to the host-parasite interactions in pertussis with particular reference to the natural history of the disease. There is evidence now to support our earlier hypothesis that pertussis is primarily a disease of adults and adolescents and secondarily a serious disease of the very young. If our hypothesis proves to be correct, then it becomes important that we interrupt the natural history of disease by reducing infectious foci in adolescents and adults. This would mean immunizing adults and adolescents and it would mean that certain surface antigens involved in the early stages of the infectious process may be more important than some of those immunogens being evaluated in the first generation parenteral vaccines.

Related to this is the prospect that vaccines can be administered by the oral route employing living or microencapsulation vectors that are capable of producing long lasting systemic and mucosal immunity. Oral administration does not require the equipment or trained personnel needed for parenteral vaccines, so that greater vaccine coverage can be achieved. It is also possible that microencapsulation may stabilize some immunogens so that there is no need for the cold chain.

But even if we had the perfect vaccine, that is to say, a vaccine that is 100 percent safe and 100 percent effective, we could not use it soon enough to protect the young infant that is most likely to suffer serious sequelae and death from pertussis. I had mentioned that the risk of disease for the infant could be reduced by immunizing adults and adolescents, but a secondary benefit of this approach may be that the newborn babies would be protected passively by vaccine-induced maternal antibodies.

With our increasing knowledge of the host-parasite interactions in pertussis, we should be able to break away from some of the classical methods of vaccine prophylaxis and develop practical methods to interrupt the natural history of this important disease. New, effective, and inexpensive methods to deliver vaccines, coupled with innovative vaccine formulations to prevent infection as well as disease, would reduce the risk of disease for the infant. Passive immunity may be used to protect the newborn infant during the first few weeks of life until a solid vaccine-induced immunity can be developed. Such an approach should result in a better control of pertussis than has been achieved with the whole cell pertussis vaccines but, most important, I think it is possible that this approach could result in the eradication of pertussis.

The **Sixth International Symposium on Pertussis** is meant to serve as a forum to review and report the results of our research and to rethink and reshape the objectives of the work that remains to be done. I welcome you all and encourage you to participate and contribute to the discussions.

Introductory Remarks

GERALD V. QUINNAN

It is an honor to welcome you all here this morning to the **Sixth International Symposium on Pertussis.** To start out, I might comment on my own viewpoint on this particular meeting. As a Government bureaucrat, I spend quite a bit of time in meetings and it is not always easy for me to tell, I have to confess, when a meeting is important and when it is not important. Some of us in that position have gotten to the point of looking around when we get into the meeting room, counting the number of heads and, based on the head count, weighing what the importance is and what it is not. By and large, that works pretty well. Looking around the room this morning, I think it is quite obvious to me that pertussis must be an important issue.

It is curious that it should be. It is more than 75 years now since pertussis vaccine was first licensed in the United States. By anybody's assessment, any scientific assessment, I think it is easy to conclude that the vaccines that are used are safe and effective, not absolutely safe and not absolutely effective, but certainly their impact on mortality and morbidity associated with pertussis infection has been dramatic.

So why are we here? I think it is a sign of the times that the public expects, and those of us in the public health community desire to produce, vaccines that are as safe and as effective as possible, and the efforts of all of you in the room are testimony to that fact.

It is also a sign of the times that there should be so much of an intense scientific effort into improving the current status of pertussis vaccination in that the technologies that are now available have so much greater potential to address this problem than what was available 10 or 20 years ago. The public often expects that with the technologies that are available that instant solutions should be available to us as well. Those in pertussis work know that instant solutions are not always the case. The technologies do give the ability, though, to understand the disease processes better and to understand the biology of this organism in a way which will make the progressive improvement of vaccines a realistic possibility.

This is the Sixth International Symposium. It is quite unusual to have multiple international conferences on a single disease in a relatively short number of years. AIDS was another example of that phenomenon, but I cannot think of many more. It is also unusual to have this many people attending a conference on a single disease in a repetitive fashion. I am confident that with the quality of the science that is being invested in this issue and the wide range of interests around the world in improving the situation that improvements will be forthcoming. I would like to congratulate all of you on the quality of the papers that have been submitted and wish you well and success in this conference. Thank you for coming.

Contents

Order Information ii

Preface iii

Opening Address. Charles R. Manclark v

Introductory Remarks. Gerald V. Quinnan, Jr. vi

Session One

Virulence Factors of *Bordetella pertussis:* Structure and Function

CO-CHAIRPERSONS: DRUSILLA L. BURNS AND WILLIAM E. GOLDMAN

Introduction. Drusilla L. Burns 3

Tracheal Cytotoxin of *Bordetella pertussis:* Biosynthesis, Structure, and Specificity. William E. Goldman,* Jayne L. Collier, Brad T. Cookson, Garland R. Marshall, and Kathryn M. Erwin 5

Structural and Functional Consequences of the Interaction of Calcium with Adenylate Cyclase Toxin from *Bordetella pertussis.* Erik L. Hewlett,* Ingrid Ehrmann, Lloyd Gray, Peter Fremgen, Margaretta Allietta, Valery M. Gordon, and Mary Gray 13

Hemolysis and Uncontrolled Intracellular cAMP Generation are Independent, Separable Functions of the Adenylate Cyclase Toxin from *Bordetella pertussis.* Arie Rogel, Ruth Meller, and Emanuel Hanski* 20

Effect of Exogenous Agents on the Action of *Bordetella* Heat-Labile Toxin. Masahiko Endoh,* Masaaki Nagai, Drusilla L. Burns, Yasukiyo Nakase, and Charles R. Manclark 30

Construction of Genetically Detoxified Pertussis Toxin. Mariagrazia Pizza, Antonello Covacci, Antonella Bartoloni, Maria Perugini, Luciano Nencioni, Roberto Manetti, Massimo Bugnoli, Paolo Ruggiero, Roberto Olivieri, Teresa de Magistris, and Rino Rappuoli* 37

Molecular Biological Studies on the Structure-Function Relationship of Pertussis Toxin and Filamentous Hemagglutinin. Camille Locht *and Teresa Cabezon 41

Pertussis Toxin and Its Mode of Entry into Eukaryotic Cells. Drusilla L. Burns,* Charles R. Manclark, and Sally Z. Hausman 53

Pertussis Toxin Substrates: Characterization of Multiple Forms of $G_{o\alpha}$ mRNA and the Requirements for Toxin-Catalyzed ADP-Ribosylation of $G_{o\alpha}$ and $G_{t\alpha}$. Mikako Tsuchiya,* M. Michael Bliziotes, Inez M. Serventi, S. Russ Price, Joel Avigan, James J. Murtagh, Jr., Linda A. Stevens, C. William Angus, Mary W. Walker, Kenneth B. Newman, Jane L. Halpern, Su-Chen Tsai, Joel Moss, and Martha Vaughan 57

Elucidation of Linear B-Cell Epitopes in the Pertussis Toxin S3 Subunit by Antipeptide Antibodies. M. Alexander Schmidt,* Bärbel Raupach, Miroslav Szulczynski, and Jutta Marzillier 66

Purification, Characterization, and Immunological Evaluation of the 69-kDa Outer Membrane Protein of *Bordetella pertussis.* Carine Capiau,* Steven A. Carr, Mark E. Hemling, Dominique Plainchamp, Karel Conrath, Pierre Hauser, Etienne Simoen, Martin Comberbach, Piet Roelants, Pierre Desmons, Philippe Permanne, and Jean O. Pétre 75

---

* Presenting author.

Session Two
Surface Proteins of *Bordetella pertussis:* Their Role as Bacterial Adhesins and Protective Antigens
CO-CHAIRPERSONS: MICHAEL J. BRENNAN AND JAMES L. COWELL

Introduction. MICHAEL BRENNAN 89

Mechanisms of *Bordetella pertussis* Adherence to Ciliated Respiratory Cells and Human Macrophages. DAVID A. RELMAN,* SAMUEL D. WRIGHT, STANLEY FALKOW, KIRSI SAUKKONEN, AND ELAINE TUOMANEN 91

Surface Proteins of *Bordetella pertussis:* Role in Adherence. ELIZABETH LEININGER,* JAMES G. KENIMER, AND MICHAEL J. BRENNAN 100

Mechanisms of *Bordetella pertussis* Invasion of HeLa 229 Cells. CAROL A. EWANOWICH,* ELIZABETH LEININGER, JAMES G. KENIMER, AND MARK S. PEPPLER 106

Invasion of HeLa 229, Chinese Hamster Ovary, and U937 Cells by *Bordetella pertussis.* CYNTHIA K. LEE,* ANGELA L. ROBERTS, THERESA M. FINN, AND JOHN J. MEKALANOS 115

Structure-Function Studies of *Bordetella pertussis* Fimbriae. A. ROBINSON,* L. I. IRONS, R. N. SEABROOK, A. PEARCE, M. MATHESON, AND S. G. P. FUNNELL 126

Repeat Sequence Motifs Constitute the Immunodominant Regions of the P.69 Protein, Pertactin from *Bordetella pertussis:* Comparison with Repeat Sequences from *Bordetella parapertussis* and *Bordetella bronchiseptica.* IAN G. CHARLES,* LI JING LI, RICHARD STRUGNELL, KATRINA BEESLEY, MICHAEL ROMANOS, GORDON DOUGAN, PAVEL NOVOTNY, IVER HERON, MARIA AU JENSEN, CHARLES R. MANCLARK, MICHAEL J. BRENNAN, AND NEIL F. FAIRWEATHER 136

Characterization of the Major Outer Membrane Porin Protein of *Bordetella pertussis.* JULIE H. HANNAH, ZHONG MING LI, MARTIN KESSEL, ALASDAIR C. STEVEN, NGA Y. NGUYEN, JOHN B. EWELL, CHARLES R. MANCLARK, AND MICHAEL J. BRENNAN* 141

Outer Membrane Vesicles of *Bordetella pertussis* Are Protective Antigens in the Mouse Intracerebral Challenge Model. JAN T. POOLMAN,* HENDRIK-JAN HAMSTRA, ANN K. BARLOW, BETSY KUIPERS, HENK LOGGEN, AND JAAP NAGEL 148

Session Three
Immunology and Host-Parasite Interactions of *Bordetella pertussis*
CO-CHAIRPERSONS: M. TERESA DE MAGISTRIS AND ROBERTA D. SHAHIN

Introduction. M. TERESA DE MAGISTRIS 159

Humoral and Cellular Immunity in Adult Volunteers Immunized with a Genetically Inactivated Mutant of Pertussis Toxin. M. TERESA DE MAGISTRIS,* A. DI TOMMASO, P. BOSSU, S. NUTI, J. OKSENBERG, A. JUDD, A. SANCHEZ, L. STEINMAN, L. NENCIONI, A. PODDA, A. TAGLIABUE, AND R. RAPPUOLI 161

Specificity of the T-Cell Response to *Bordetella pertussis* in Aerosol-Infected Mice. KINGSTON H. G. MILLS,* AMANDA BARNARD, JOHANNA WATKINS, AND KEITH REDHEAD 166

---

* Presenting author.

Cell-Mediated Immune Responses in Patients with Pertussis and in Asymptomatic Exposed Contacts. Dean A. Blumberg,* Evelyn Pineda, Linda P. Smith, Patricia Chatfield, ChrisAnna M. Mink, Peter D. Christenson, and James D. Cherry . . . 175

Immunoglobulin E Response to *Bordetella pertussis* Filamentous Hemagglutinin and Pertussis Toxin in Mice. Diane S. J. Lindsay, Roger Parton, and Alastair C. Wardlaw . . . 179

Immunoglobulin E Response to Pertussis Toxin after Vaccination with Acellular Pertussis Vaccine. Margareta Blennow,* Marta Granström, and Bengt Björkstén . . . 184

Live Oral Attenuated *Salmonella typhimurium* Vaccine Vectors Which Induce Formation of Antibody to *Bordetella pertussis.* Charlotte D. Parker,* N. Christine Molina, Sandra M. Kelly, Roy Curtiss III, and Jinan Yu . . . 189

Analysis of Protective Mucosal Immunity to Respiratory *Bordetella pertussis* Infection. Roberta D. Shahin,* Diana F. Amsbaugh, Mayumi Endoh, and Charles R. Manclark . . . 196

Investigation of the Role of the *Bordetella pertussis* Virulence Factors in Disease. Alison Ann Weiss,* Matina St. Mary Goodwin, and Neil Allison . . . 202

Bordetella pertussis Adenylate Cyclase: A Protective Antigen against Lethality and Bacterial Colonization in Murine Respiratory and Intracerebral Models. Nicole Guiso,* Marek Szatanik, and Murielle Rocancourt . . . 207

Session Four
Molecular Genetics and Regulation of Virulence Factors
CO-CHAIRPERSONS: CAMILLE LOCHT AND E. SCOTT STIBITZ

Introduction. E. Scott Stibitz . . . 215

Regulation of *fhaB, bvg,* and *ptx* Transcription in *Escherichia coli:* a Comparative Analysis. Jeff F. Miller, Craig R. Roy,* and Stanley Falkow . . . 217

Genetic Studies on the *vir* Locus of *Bordetella pertussis.* Scott Stibitz* and Mei-Shin Yang . . . 225

Regulation and Functional Characterization of *Bordetella pertussis* Adenylate Cyclase-Hemolysin. Sophie Goyard, Jacques Bellalou, Hiroshi Sakamoto, Daniel Ladant, Brid M. Laoide, and Agnes Ullmann* . . . 233

A Strategy for Studying Pathogenic Mechanisms: Transposon Insertion Mutations with Conditional Phenotypes. Brad T. Cookson,* Tihamer Tomcsanyi, Douglas E. Berg, and William E. Goldman . . . 243

Production of *Bordetella pertussis* Fimbriae: Sequences Involved in Regulation and Identification of an Accessory Gene. Frits R. Mooi,* Rob J. L. Willems, and Han G. J. Van Der Heide . . . 251

Mutational Analysis of the Adenylate Cyclase Toxin of *Bordetella pertussis.* Eileen M. Barry,* Alison A. Weiss, Mary C. Gray, Erik L. Hewlett, Ingrid E. Ehrmann, and Matina S. Goodwin . . . 260

Three Sites Upstream of the *cya* Operon Are Required for Phenotypic Modulation and Hemolytic Activity of the Adenylate Cyclase from *Bordetella pertussis.* Kirsi Saukkonen, Stephen Sande, and H. Robert Masure . . . 267

P.69 Pertactin: High-Level Expression and Purification from *Escherichia coli* and Protective Properties. Neil F. Fairweather,* A. J. Makoff, M. D. Oxer, S. Ballantine, M. Roberts, Gordon Dougan, and Ian G. Charles . . . 275

---

* Presenting author.

Autoregulation of the Expression of the Gene for Pertussis Toxin S1 Subunit in *Escherichia coli.* YVES LOBET* AND WITOLD CIEPLAK, JR. 281

Session Five

Pertussis Epidemiology, Diagnosis, and Clinical Studies

CO-CHAIRPERSONS: BRUCE D. MEADE AND C. H. WIRSING VON KÖNIG

Introduction. BRUCE D. MEADE 289

Evaluation of Laboratory Methods Used for the Diagnosis of Pertussis Infection and Disease in the Swedish Efficacy Trial. JANN STORSAETER,* HANS O. HALLANDER, AND CONOR P. FARRINGTON 291

Acellular Pertussis Vaccine Efficacy Determined from Clinical Criteria. WILLIAM C. BLACKWELDER 295

New Conclusions and Lessons Learned from the Vaccine Trial in Sweden. PATRICK OLIN 299

Preliminary Comparison of Antibody Responses and Symptoms Following Primary Immunization with British Whole-Cell and Three Acellular DTP Vaccines. E. MILLER, D. L. MILLER,* L. A. E. ASHWORTH, P. A. WAIGHT, AND K. A. HARBERT 303

Outstanding Issues in the Clinical Evaluation of New Acellular Pertussis Vaccines. ROGER H. BERNIER 311

Detection of *Bordetella pertussis* in Clinical Specimens. C. H. WIRSING VON KÖNIG,* JÖRG E. HOPPE, ANGELIKA TACKEN, AND HORST FINGER 315

Serodiagnosis of Pertussis. BRUCE D. MEADE,* CHRISANNA M. MINK, AND CHARLES R. MANCLARK 322

Nasal Immunoglobulin A Responses To Pertussis Toxin, Filamentous Hemagglutinin and Agglutinogens 2 and 3 after Infection with *Bordetella pertussis* and Immunization with Whole-Cell Pertussis Vaccine. MARK G. THOMAS,* L. A. EDWARD ASHWORTH, ELIZABETH MILLER, AND HAROLD P. LAMBERT 330

Epidemiology of Pertussis in Philadelphia. SARAH S. LONG,* MARGARET C. FISHER, JOEL E. MORTENSEN, AND ADAMADIA DEFOREST 335

Poster Session Contributions 347

List of Participants and Contributors 357

Participant and Contributor Index 393

Subject Index 401

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* Presenting author.

SESSION ONE

Virulence Factors of *Bordetella pertussis*: Structure and Function

Co-chairpersons:

Drusilla L. Burns
William E. Goldman

Introduction

DRUSILLA L. BURNS

The first session deals with the structures and function of the virulence factors produced by *Bordetella pertussis*. As we all know, a number of molecules may be involved in the pathogenicity of this organism and I have divided these into two classifications: toxins and adhesins. *Bordetella pertussis* produces numerous toxins, and we will talk about four of these today: tracheal cytotoxin, adenylate cyclase toxin, heat labile toxin, and pertussis toxin. Since *Bordetella pertussis* produces many toxins which may be involved in the disease, we need to know not only about each of the individual toxins, but eventually we are going to have to know about the complex interactions that may occur between these toxins which also play a role in disease.

The colonization of the host also appears to be a complex process, since there are multiple adhesins that may be involved in attachment of the organism to host tissues. These include filamentous hemagglutinin, 69-kDa protein and pertussis toxin which also have been reported to be adhesins.

Each of these molecules has now been purified, which allows us to study them at the molecular level. Today we're going to hear about some of the latest studies.

Tracheal Cytotoxin of *Bordetella pertussis:* Biosynthesis, Structure, and Specificity

WILLIAM E. GOLDMAN,[1]* JAYNE L. COLLIER,[1] BRAD T. COOKSON,[1]
GARLAND R. MARSHALL,[2] AND KATHRYN M. ERWIN[1,2]

Departments of Molecular Microbiology[1] and Pharmacology,[2] Washington University School of Medicine, St. Louis, Missouri 63110

The most recently discovered toxic component of *Bordetella pertussis* is tracheal cytotoxin (TCT), a molecule completely unlike conventional exotoxins of bacteria. At 921 daltons, this toxin is among the smallest bacterial products known to generate cytopathological effects. TCT is derived from peptidoglycan, a nearly ubiquitous macromolecule among the prokaryotes. Yet its activity is remarkably selective: TCT is responsible for the specific ciliated cell damage that disables the primary mechanical defense barrier in the lung. The violent coughing episodes so characteristic of pertussis are desperate attempts to clear the stagnant airways of accumulating mucus, bacteria, and the products of airway inflammation (for a review, see reference 7).

Two in vitro models led to the discovery of TCT: (i) hamster tracheal organ cultures, in which TCT causes ciliostasis and ciliated cell extrusion, and (ii) hamster trachea epithelial (HTE) cell cultures, which stop multiplying after exposure to TCT (8). We have suggested previously that the effect on nonciliated HTE cells may reflect an impairment of precursor basal cell division and differentiation to replace ciliated cells. Thus, release of TCT by *B. pertussis* would not only destroy the mucociliary clearance mechanism, but would prevent its repair by a long-term inhibition of epithelial regeneration. This could certainly explain the continuation of the pertussis syndrome long after antibiotics have been administered and the organisms can no longer be cultured (16).

Recently, methods were developed for the rapid purification of this biologically active material from the culture supernatant of *B. pertussis* (4). Completely purified TCT was then structurally characterized (3) as *N*-acetylglucosaminyl-1, 6-anhydro-*N*-acetylmuramyl-alanyl-γ-glutamyl-diaminopimelyl-alanine (see Fig. 1). This characterization makes TCT a member of the muramyl peptide family, which includes the well-studied muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine). The diverse biological activities of muramyl peptides include adjuvanticity, arthritogenicity, stimulation of leukocytes to produce interleukin-1, and somnogenicity (1, 2, 9).

The mechanism by which TCT can generate such dramatic cytopathology in the respiratory tract is not yet understood. Because this tiny fragment of peptidoglycan obviously lacks enzymatic activity, we have hypothesized that TCT's specific toxicity is a result of its interaction with specific host receptors or target sites. Consequently, there should be portions of the TCT molecule where the precise structure is essential for respiratory epithelial binding and/or toxicity. This report presents several lines of evidence consistent with this theory.

MATERIALS AND METHODS

Purification and analysis of TCT. TCT was purified from the supernatant of *B. pertussis* Tohama I cultures by using a combination of solid-phase extraction and reversed-phase high-pressure liquid chromatography (HPLC) as described previously (4). Amino acid analysis was performed for quantitation and to check purity. Biological activity was monitored in the two systems described previously: (i) generation of ciliated cell-specific damage in hamster tracheal rings and (ii) inhibition of DNA synthesis in cultures of HTE cells (4).

Assay for TCT production. To quantitate TCT production on an analytical level, *B. pertussis* culture supernatants were processed through the first C_{18} solid-phase extraction step of the purification protocol mentioned above. After drying the samples on a Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, N.Y.), they

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* Presenting author.

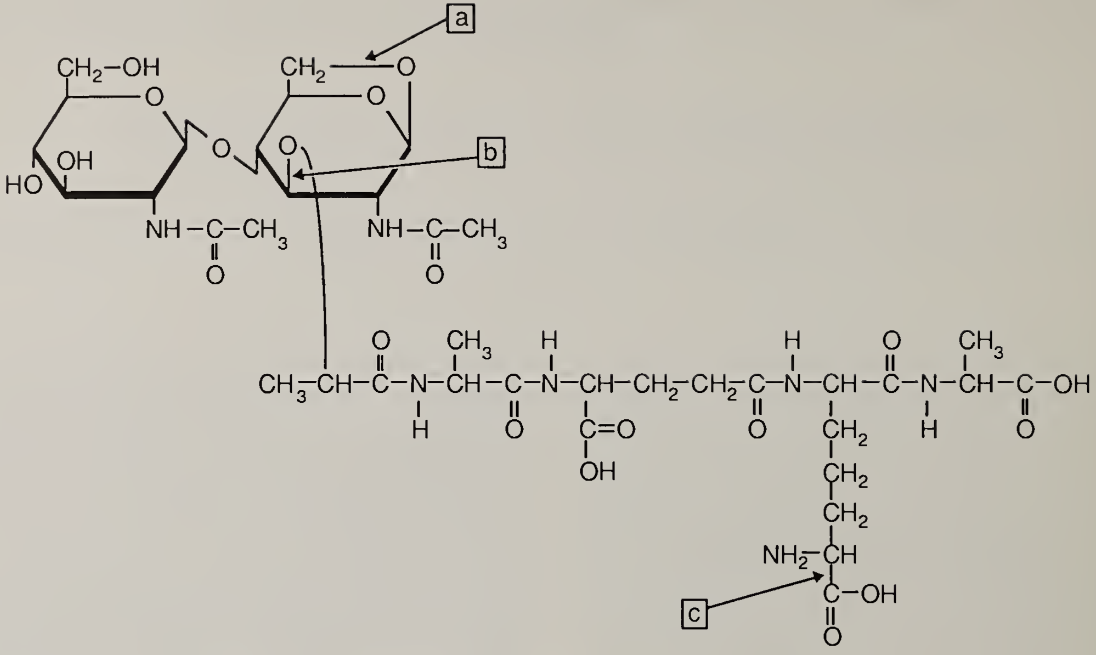

FIG. 1. TCT structure, indicating bonds that were altered in analogs used for this study. (a) is hydrolyzed to yield the reducing monomer of peptidoglycan, (b) is cleaved by alkali-catalyzed β-elimination to generate the TCT lactyl tetrapeptide, and (c) shows the further decarboxylation represented in the gram-positive lactyl tetrapeptide.

were reacted with methanol:water: triethylamine:phenylisothiocyanate (75:10:10:5) for 1.5 h at room temperature. This reaction of phenylisothiocyanate with TCT forms phenylthiocarbamyl-TCT (PTC-TCT). Excess reagents were removed by evaporation, and the samples were chromatographed on a Spheri-5 RP-8, 250 x 4.6 mm cartridge column (Applied Biosystems, San Jose, Calif.) maintained at 35°C. A gradient was developed starting with 92% solution A (25 mM sodium acetate, 0.05% triethylamine, pH 6.35) and 8% solution B (60% acetonitrile). This chromatography system distinctly separated PTC-TCT from other derivatized molecules in supernatant extracts. Individual samples were quantitated by comparison to the A_{254} of PTC-TCT standards. The amount of TCT produced was compared to the amount of *B. pertussis* growth as measured by the A_{540} of the broth culture.

Generation of TCT analogs. TCT is the anhydro monomer of gram-negative peptidoglycan and can be isolated from *B. pertussis* culture supernatant as mentioned above. A closely related TCT analog is the reducing monomer which lacks the internal dehydration between the first and sixth carbons of muramic acid (Fig. 1). To purify this molecule, peptidoglycan of *B. pertussis* Tohama III was isolated by cold trichloroacetic acid precipation, sodium dodecyl sulfate treatment, and ultracentrifugation (5). The pellet was treated with lysozyme to generate reducing monomers which were then isolated by preparative reversed-phase HPLC methods like those used for TCT purification. Another TCT analog was generated by treating these reducing monomers with aqueous ammonia, which removes the disaccharide portion (21). The resulting lactyl tetrapeptide (Fig. 1) was also purified by reversed-phase HPLC. Finally, a lactyl tetrapeptide analog, lac-L-ala-D-γ-glu-L-lys-D-ala, was prepared by chemical synthesis. Commerically available amino acids with appropriate protections and free D-lactic acid were used in solid-phase peptide synthesis on Merrifield resin using BOP (benzotriazolyl-*N*-oxy-tris(dimethylamino)phosphonium hexafluorophosphate) as a coupling agent (6, 14). For all analogs, structures and purity were confirmed by FAB-MS and quantitation was by amino acid analysis. MDP and diaminopimelic acid (DAP) were obtained from Sigma Chemical Co., St. Louis, Mo.

Radioligand binding studies with HTE cells. HTE cells were seeded at a density of 3 x 10^5 cells per 35-mm diameter well using plastic pretreated with poly-L-lysine (100 μg/ml) to improve cell adherence. Cells were incubated in minimal essential medium (GIBCO Labora-

TABLE 1. Toxicity of muramyl peptides for respiratory epithelial cells

Muramyl peptide	Structure	Toxicity for: HTE cells[a]	Toxicity for: Tracheal rings[b]
TCT (anhydromonomer)	GlcNac-1,6-anhydro-MurNac-L-ala-D-γ-glu-*meso*-DAP-D-ala	+	+
Reducing monomer	GlcNac--------------- MurNac-L-ala-D-γ-glu-*meso*-DAP-D-ala	+	+
MDP (Muramyl dipeptide)	MurNac-L-ala-D-isogln	–	–
TCT lactyl tetrapeptide	Lactyl-L-ala-D-γ-glu-*meso*-DAP-D-ala	+	+
Gram-positive lactyl tetrapeptide	Lactyl-L-ala-D-γ-glu-----L-lys-----D-ala	–	–
Diaminopimelic acid (mixture of stereoisomers)	DAP	–	–

[a] "+" toxicity for HTE cells indicates the ability to cause a dose-dependent inhibition of DNA synthesis, using concentrations comparable to TCT's normal toxic levels (half-maximal toxicity of 0.3 to 0.4 μM).
"-" indicates that even much higher doses (usually 100 x) had no corresponding effect.

[b] "+" toxicity for hamster tracheal rings indicates the development of ciliostasis and ciliated cell specific damage and extrusion, generally at doses around 3 μM.

tories, Grand Island, N.Y.) with 2.5% FBS (HyClone, Logan, Utah) overnight at 37°C in 95% air-5% CO_2. The incubation medium was removed and the cells were washed with phosphate-buffered saline containing 11.1 mM glucose, 0.5 mM $MgCl_2$, 0.14 mM $CaCl_2$, and 0.28 mM ascorbic acid. ^{3}H-serotonin (25 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to wells at a concentration of 20 nM along with titrations of TCT as a competitive ligand. After 1 h incubation at 37°C, the monolayers were washed, solubilized with 0.5N NaOH, and assayed for bound radioactivity in a scintillation counter. Controls with an excess of unlabeled serotonin (5 μM) in place of TCT were included to measure the amount of specific binding.

RESULTS AND DISCUSSION

TCT production by *B. pertussis*. TCT is a monomeric fragment of peptidoglycan released into the supernatant of *B. pertussis* cultures. Enzymatic processing of intact peptidoglycan is an activity common to all prokaryotes with a cell wall; presumably, specialization of this process allows production of TCT. Peptidoglycan turnover in *B. pertussis* is not unusually high (18), yet TCT accumulates at levels over 1 μM in broth cultures (4). A possible explanation is that *B. pertussis* production of TCT relies on autolysis and that we have merely purified one of many peptidoglycan fragments released from dead and dying organisms. To evaluate TCT production during *B. pertussis* growth, a sensitive assay was developed to detect TCT in culture supernatants. By derivatizing supernatant extracts with phenylisothiocyanate and separating the reaction products by HPLC, levels of TCT as low as 15 pmol can be rapidly quantitated.

Time-course experiments revealed that TCT is released only by growing *B. pertussis:* accumulation of TCT in the culture supernatant ceased as bacteria reached stationary phase (Fig. 2). At this stage, an increase in TCT release might be expected if production were simply the result of cell lysis and the corresponding breakdown of peptidoglycan. In addition to these observations, earlier pulse-chase studies (using ^{3}H-DAP to monitor peptidoglycan metabolism) showed that TCT accounts for >90% of the peptidoglycan released by log-phase *B. pertussis* (18). In fact, *B. pertussis* peptidoglycan has a remarkably uniform structure, with disaccharide-tetrapeptides making up >95% of the monomer subunits (5). Thus, intact *B. pertussis* cell wall appears to be the ideal substrate for generating homogeneous peptidoglycan fragments by enzymatic processing.

Together, these results imply that TCT production is not merely the result of cell deterioration and autolytic breakdown of peptidoglycan into small fragments. Instead, TCT appears to be a specific product of a carefully regulated metabolic process. Other gram-negative organisms such as *Escherichia coli* and *Alcaligenes faecalis* (the most closely related species to *Bordetella*)

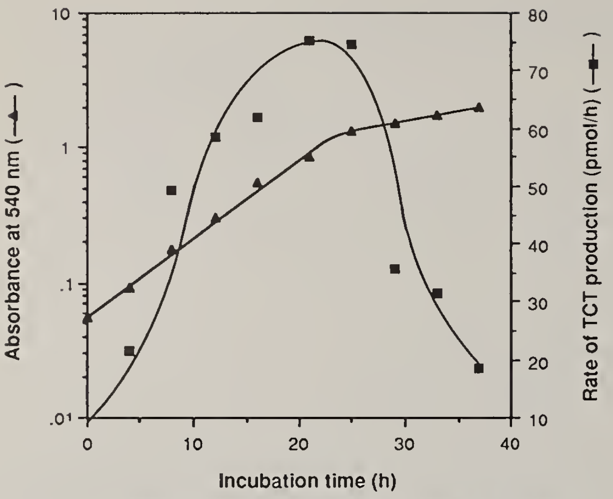

FIG. 2. TCT production by *B. pertussis*. Culture supernatants were assayed for the presence of TCT at various time points during broth culture growth of *B. pertussis*.

are unable to produce detectable TCT in their culture supernatants (unpublished results). One organism that does release peptidoglycan fragments in large amounts is *Neisseria gonorrhoeae*, which releases TCT-like molecules able to generate ciliated cell-specific damage in fallopian tube mucosa (13).

Interestingly, *N. gonorrhoeae* produces a rather heterogeneous collection of peptidoglycan fragments (20), unlike the nearly singular release of TCT by *B. pertussis*.

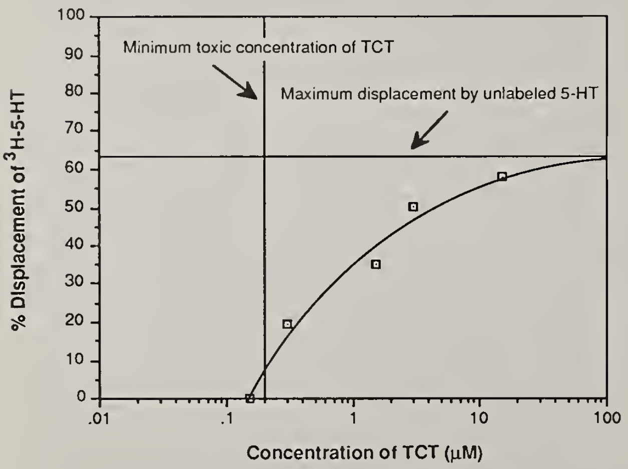

FIG. 3. TCT displacement of ^{3}H-5-HT binding to HTE cells. The horizontal line indicates the maximum amount of specific binding of ^{3}H-5-HT, based on the ability of a vast molar excess of unlabeled 5-HT to compete. The vertical line indicates the usual TCT concentration at which toxicity for HTE cells can begin to be measured (4). In this experiment, physiological doses of TCT were able to interfere with nearly all the specific binding of ^{3}H-5-HT.

Because of TCT's role in disabling host clearance mechanisms (by eliminating ciliary activity) and in facilitating transmission (by coughing), the ability to produce TCT efficiently has probably been highly favored during *B. pertussis* evolution. This also suggests that the specific structure of TCT is important, since *B. pertussis* is not relying on a random breakdown of peptidoglycan that would generate an assortment of small fragments. This hypothesis is further supported by studies with defined TCT analogs, as described below.

TCT structure-function relationships. In studies of the biological activities of other muramyl peptides, structural analogs have been important in defining the minimal active structure as well as in identifying functionally important features. For example, MDP is a synthetic muramyl peptide that defines the minimal structure required for the immunological effects of Freund's complete adjuvant (1). We prepared a number of TCT analogs to evaluate how specific structural features contribute to the toxin's effects on respiratory epithelium. The results in Table 1 show how variations in structure consistently affected toxicity in both model systems.

The reducing monomer, identical to TCT except for a single hydrolysis of the internal bond between the first and sixth carbons of muramic acid (Fig. 1), showed TCT-like toxicity for respiratory epithelial cells. This finding is in contrast to studies on the slow-wave sleep-promoting effects of peptidoglycan (10); in those experiments, the anhydro monomer was a much more potent somnogen. MDP was not toxic in our system, in contrast with the dramatic effects of that analog on immune responsiveness. The results, however, were consistent with the toxicity for fallopian tube ciliated cells, which are also affected to the same extent by both anhydro and reducing monomers but are unharmed by MDP (13).

The most surprising result was with a TCT analog that completely lacks the sugars of the native molecule. This lactyl tetrapeptide had a dose response identical to TCT's inhibition of HTE cell division, and its effects on tracheal rings were indistinguishable from those of TCT. Therefore, the sugar portion is apparently not required for TCT activity, in sharp contrast to the other biological effects of muramyl peptides (11). This finding implies that TCT toxicity for respiratory epithelial cells is mediated through a different receptor or mechanism.

Within the peptide portion, DAP appears to be an especially critical residue for TCT function. The gram-positive lactyl tetrapeptide differs in only one respect from the TCT lactyl tetrapeptide: the substitution of lysine for DAP means the loss of the sidechain carboxyl group (Fig. 1). Yet this minor change caused more than a 100-fold drop in toxicity. DAP alone, however, was not sufficient to generate the toxic effects of TCT.

Thus, the respiratory epithelial toxicity of TCT is very different than the previously described activities of muramyl peptides. Tests of structural analogs indicated that the sugars of TCT are unimportant for toxicity, though they may confer an important role in vivo (e.g., resistance to degradation by host enzymes). However, the impact of even minor structural variations within the TCT peptide portion indicates that there are precise structural features essential for toxicity. Our initial hypothesis was that fine structural requirements for toxicity implied that TCT must bind to a specific receptor or target site. As described below, however, a cell surface receptor may be involved in TCT action.

TCT binding to respiratory epithelial cells. Muramyl peptides cause a remarkable variety of responses, depending on the target cells or tissues involved. A study by Silverman et al. (19) demonstrated that muramyl peptides compete with the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) for binding sites on macrophages. Previous research suggested that at least some of the biological effects of muramyl peptides are mediated via serotonergic mechanisms (12) and that a shared binding site may explain the role of both in modulating sleep (17). Although structural similarities between TCT and 5-HT are not obvious, this possible explanation for the effects of TCT on the respiratory epithelium was intriguing, and we designed similar competition experiments with HTE cells.

These studies monitored ^{3}H-5-HT binding to intact HTE cell monolayers. Co-incubation with high levels of unlabeled 5-HT displaced 63% of labeled ligand binding, indicating that 37% of the binding is nonspecific or that some of the ^{3}H-5-HT has been internalized (Fig. 3). TCT, when incubated along with ^{3}H-5-HT, was also able to displace as much as 58% of radioligand binding to HTE cells. The inhibition of ^{3}H-5-HT binding was apparent at TCT concentrations that generate toxicity in respiratory epithelial cells (Fig. 3). These data imply that (i) a specific 5-HT binding site is present on HTE cells and (ii) this putative 5-HT receptor is able to bind TCT.

Similar results were seen in experiments at 4°C, which minimized ligand internalization and the indirect effects on target cell metabolism. However, these studies required longer incubation times and were often compromised by the extreme lability of 5-HT, which is readily oxidized or is degraded by an apparent monoamine oxidase produced by HTE cells. Certain conditions also revealed the presence of an additional 5-HT binding site which does not bind TCT. Therefore, more exacting binding studies, including saturation, kinetic, and equilibrium analyses, will require the use of more stable 5-HT analogs as well as some refinements to the assay conditions.

It is not yet clear whether the 5-HT binding site is the only TCT binding site on respiratory epithelial cells. Judging from these competition experiments, TCT has a rather low affinity for this site, although this affinity corresponds to the molar concentrations required for toxicity. Furthermore, it is not known if binding to this putative 5-HT receptor is responsible for TCT toxicity. Nevertheless, these results are consistent with the hypothesis that TCT may act by mimicking or interfering with 5-HT effects on target cells. The distribution of this 5-HT binding site would dictate the specificity of TCT effects on target tissue, and side effects (e.g., sleep) could be mediated by 5-HT receptors in other tissues.

Conclusions. We initially proposed that the precise structure of TCT is important in binding to and/or toxicity for respiratory epithelial cells. The studies described here have shown that (i) TCT is a specific and nonrandom product of actively growing *B. pertussis;* (ii) certain portions of the TCT disaccharide-tetrapeptide structure are critical for toxicity; and (iii) TCT interferes with the specific binding of 5-HT to respiratory epithelial cells. All of these studies therefore point to the importance of specific TCT structure as the basis for its toxicity.

This finding may seem to be a wholly unconventional view of peptidoglycan, a nearly universal prokaryotic structure which is usually only thought to provide rigidity for the cell wall. The concept is not surprising, however, considering the remarkable array of biological activities of muramyl peptides. *B. pertussis* appears to have evolved a mechanism for using peptidoglycan to its advantage in establishing a disease state and ensuring its own transmission via coughing. Other *B. pertussis* virulence determinants also contribute to the process of TCT intoxication: adhesins that enable bacterial binding to ciliated cells, making TCT delivery more efficient; and toxins that interfere with immune response cells so that TCT may be delivered for a longer period of time. In fact, pertussis toxin's well-known "histamine-sensitization" activity (15) makes animals much more sensitive to the effects of subsequently administered 5-HT (which is a structural relative of histamine). Although the mechanism remains unknown, it is intriguing that pertussis toxin could be responsible for making the host much more responsive to the effects of TCT.

ACKNOWLEDGMENTS

This work has been supported by Public Health Service grant AI22243 from the National Institutes of Health, by the Monsanto/Washington University Biomedical Research Contract, and by Public Health Service training grants AI07172, GM07805, and GM07200 from the National Institutes of Health to Washington University School of Medicine.

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DISCUSSION

DR. POOLMAN: (Inaudible) . . . If I remember correctly, the studies looked into two models. There was a great proportion of the sloughing and stopping of ciliation that was caused by endotoxin. How is that working in your system?

DR. GOLDMAN: That is right. They showed very nicely in actually companion studies that lipopolysaccharide could duplicate the kinds of effects they were seeing with the peptidoglycan monomers. We have tried lipopolysaccharide, both from *Bordetella* and from *E. coli*, and we have put it into both of our model systems. At extremely high doses we can see damage by LPS alone in the tracheal organ culture system but the doses that were active exceeded, I think it was 50 micrograms per milliliter when we were doing those experiments. That was very, very high. That exceeds the amount that we would find in culture supernatants.

When we saw that damage, I should point out also, that we did not see ciliated cell-specific damage. We saw total cellular destruction of all the cells in the epithelium. There was simply nothing left so we did not see that in the respiratory epithelial system, we did not see that mimicking the same kind of pathology as natural pertussis infection. In the other model that we use with hamster trachea epithelial cells, it also takes very high doses to see any sort of damage, and, in fact, at some lower doses we see stimulation of growth by LPS rather than any sort of interdiction.

DR. POOLMAN: Did you study meningococcal polysaccharide?

DR. GOLDMAN: We have not looked at that.

DR. HABIG: Do you believe you could make an immunogenic derivative of TCT that might be useful as a vaccine component?

DR. GOLDMAN: That is a particularly sore point with me because we tried for quite a while to generate antibodies in a number of systems. We did it by trying to conjugate TCT to a number of carriers using a number of different linkages. We did it in hamsters and rabbits and mice. We even implanted chambers with *Bordetella* growing in the peritoneal cavity trying to get *Bordetella* to naturally produce TCT. In not one of these instances, and my poor post-doc suffered through this, did we generate any antibody. In talking with other laboratories that worked with small pieces of peptidoglycan, muramyl peptides, they have confirmed that they also are unable to generate any antibodies in their work. One group has tried for five years, and their feeling and the general hypothesis right now is that animals in general are made tolerant to peptidoglycan very early in life just by their exposure to food. Even germ-free animals, if you chose to go that route, would be exposed to bacteria in their autoclaved food so it is generally thought now by all of us who have tried and crashed and burned that it is nearly impossible to make antibodies to these molecules but, of course, I am open to any suggestions.

DR. FRIEDMAN: Bill, it was a very interesting talk. In the assays you look at inhibition of DNA synthesis. Is that the site of toxic effect of TCT or do you have a feeling now as to what is the specific initial site for toxicity?

DR. GOLDMAN: I do not think I have any better feeling for that right now. One thing that you point out, I think, is that ciliated cells do not divide so we have one model where we look at destruction of ciliated cells and another model where we look at the inhibition of DNA synthesis in a dividing cell population. We do not know what the two have to do with each other. They may, in fact, have nothing to do with each other. The only advantages, the cells we use, the tracheal cells, are derived directly from hamster tracheal organ cultures so we know that they are genetically related but we do not really know what the two effects have to do with each other. Our speculation has always been that the effect on the proliferative activity of the tracheal epithelial cells may reflect a similar or analogous effect on tracheal epithelium in vivo where the replacement population of cells, the basal cells, might be inhibited in terms of them replacing lost ciliated cells. That is the normal functional regeneration in the respiratory tract. I do not have anything more concrete to tell you.

DR. PEPPLER: I was wondering if you could compete the activity of TCT in your hamster epithelial cell system with your synthetic lacteal, I believe it was streptococcal cell wall component.

DR. GOLDMAN: The gram positive lysine equivalent, we have not done binding type competition studies but we did do a biological activity study where we threw in excess, tetrapeptide to try to compete the biological activity of TCT and that did not show any competition.

DR. HEWLETT: Bill, it was a very nice presentation. Have you begun to address the issue of cleavage of the peptidoglycan and the regulation of the release of this specific molecule? Your time course certainly suggests the possibility that that is a process that is turned on and then turned off again.

DR. GOLDMAN: Right, Brad Cookson has done some of these studies and is really better prepared to talk about them than I am, but what he has found is that under all sorts of conditions that regulate growth of *Bordetella*, you correspondingly regulate TCT production. If you can do anything to slow down growth, you slow down TCT production and vice versa. We have not looked at any more of the specifics about the particular enzymes involved.

DR. BURNS: You said that serotonin can affect the cough reflex. Can you speculate wildly what TCT might do as far as coughing is concerned and what effect TCT might have if it is bound?

DR. GOLDMAN: In the studies that I have seen which looked at cough reflex regulation by serotonin, what serotonin does is to actually suppress the cough reflex. When you have a normal level of serotonin in the brain, you suppress coughing. So if I am allowed to speculate here about brain tissue, which I have never worked with, if TCT is competing with serotonin for binding to that particular receptor, then one might expect that you would alleviate or relieve the kind of suppression that serotonin might normally do if TCT has an antagonistic sort of function and then you might trigger coughing as a result. I am not sure.

Structural and Functional Consequences of the Interaction of Calcium with Adenylate Cyclase Toxin from *Bordetella pertussis*

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Bordetella pertussis produces an adenylate cyclase (AC) which is capable of interacting with and entering eukaryotic cells whereupon it is activated by calmodulin to produce cAMP from available host ATP (5, 9, 14). This intoxication of cells by AC toxin has been demonstrated to occur in a variety of cell types, with consequences generally commensurate with the known role of cAMP in that cell (5, 6, 19-21). In several of these studies, it was noted that toxin activity was markedly reduced in the absence of extracellular calcium (7, 12, 18). In addition, Gordon et al. (10) demonstrated the ability of AC toxin to release marker from multilamellar liposomes and showed that the activity was calcium-dependent. More recently, Glaser et al. (8) cloned and sequenced the structural gene for AC toxin (*cyaA*), noting a partial sequence homology with *Escherichia coli* hemolysin. This homology included a region containing a repeating motif, GGDGDDTLX, which is present in other bacterial hemolysins (16) and was suggested by Welch et al. (22) to resemble calcium-binding domains in other proteins. Using purified AC toxin (13), it has been demonstrated that the 216-kDa holotoxin molecule lyses sheep erythrocytes in a calcium-dependent manner, but with a delayed onset and slower time course than those of *E. coli* hemolysin (E. L. Hewlett, I. E. Ehrmann, M. C. Gray, W. W. Young, Jr., and V. M. Gordon, *in* R. Rappuoli, F. Fehrenbach, J. Freer, P. Falmagne, J. Alouf, R. Gross, M. Tomas, C. Montecucco and T. Wadstom (ed.), *Protein Bacterial Toxins*, in press). As with *E. coli* hemolysin (1), the AC toxin appears to produce a pore in the target RBC membrane but one which is approximately fivefold smaller.

Adenylate cyclase enzyme activity of the toxin is activated by calmodulin, but remarkably this can occur in the absence of calcium (11, 15). The other activities of AC toxin (target cell intoxication, liposome lysis, and hemolysis) are, however, calcium-dependent (7, 10, 12, 18). Although effects of calcium on the biophysical properties of AC have been studied by several groups, the role of calcium, in biological activities remains unclear. This project investigated the interaction of purified AC toxin with calcium, the results of those studies are presented here.

MATERIALS AND METHODS

B. pertussis culture and AC toxin production. *B. pertussis* strain BP338 was grown as described previously (12). The AC toxin was purified by urea extraction, hydrophobic chromatography, preparative sucrose gradient centrifugation, and calmodulin-Sepharose affinity chromatography as described (13).

Assays for biological activities of AC toxin. (i) Enzymatic activity was measured by quantitation of the conversion of ^{32}P-ATP to ^{32}P-cAMP in the presence of 1.0 µM of calmodulin as described (13).

(ii) Jurkat cells, human T-helper cells, were maintained in RPMI 1640 medium supplemented with 15% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin in 5% CO_2. Toxin activity was assayed in Jurkat cells after incubation with AC toxin at 37°C for 0.5 h (13). Quantitation of the intoxication of sheep erythrocytes was complicated by the presence of hemoglobin which needed to be removed by trichloroacetic acid (TCA) precipitation. Cyclic AMP remained in the supernatant medium which was ex-

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TABLE 1. Effect of calcium concentration on the enzyme toxin, and hemolytic activities of AC toxin

[Ca++] (M)	Enzyme[a] (pmol/min per μg)	Toxin (pmol/mg cell prot per μg)	Hemolysis (%)
3.60×10^{-8}	45,200 ± 2,900	13.8 ± 2.3	6.2
1.25×10^{-3}	44,000 ± 1,730	2,030 ± 34	53

[a] Values are mean ± SD.

tracted with H_2O-saturated ether to remove residual TCA prior to quantitation of cAMP by automated radioimmunoassay (2, 3).

(iii) Hemolytic activity (Hewlett et al., in press) was determined according to the method of Clerc et al. (4) using washed sheep erythrocytes at a concentration of 4 x 10^9/ml in phosphate-buffered saline. The assay was carried out in 96-well microtiter plates at 37°C for 5 h. Values for hemolysis are expressed as a percentage of total hemolysis elicited by addition of ammonium hydroxide.

TABLE 2. Effect of Ni on enzyme, toxin, and hemolytic activities of AC toxin

Addition	Enzyme (pmol/mg per μg)	Jurkat cells	Sheep erythrocytes	
		Toxin (pmol/mg per μg)	Toxin (pmol/2 x 10^8 cells)	Hemolysis (%)
None	79,200	2,630	885	79.8
Ni (5mM)	66,700	24.2	221	80.8

Interaction of calcium with AC toxin. (i) Calcium binding was determined (17) by incubation of AC toxin and calmodulin (control) with ^{45}Ca after processing with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to polyvinylidene difluoride membranes. After incubation with ^{45}Ca (1 μCi/ml) for 30 min, the membranes were washed and used to expose x-ray film.

(ii) The fluorescence emission spectrum of AC toxin at a concentration of 70 μg/ml was measured in an SLM 8000 spectrofluorimeter. The sample was placed in a quartz cuvette with the sample chamber maintained at 4°C. Excitation wavelength was 295 nm and the emission spectrum over the range of 300 nm to 450 nm in 1-nm increments was recorded. Bandpass was set at 4 nm for excitation and emission monochrometers. Data were normalized and expressed as fluorescence intensity.

(iii) Electron microscopy was carried out on samples of affinity-purified AC toxin. The toxin proteins were negatively stained for electron microscopy by floating a thin carbon film onto a droplet of approximately 0.03 mg/ml protein for 1 min, briefly washed on a droplet of buffer, and finally transferred to a droplet of 0.5% aqueous uranyl formate for 30 s. The carbon film was picked up on a 300-mesh nickel grid and allowed to air dry for viewing with a Zeiss 902 electron microscope.

RESULTS

As shown in Table 1, the enzymatic activity of AC toxin is the same at 36 nM and 1.25 mM free-calcium; this indicates that in the presence of a maximal concentration of calmodulin (1 μM), calcium is not a determinant of this activity. In contrast, the abilities of AC toxin to enter Jurkat cells and produce intracellular cAMP and to lyse sheep erythrocytes are essentially absent at 36 nM free-calcium. Similarly, the liposome-disrupting activity of AC toxin was shown by Gordon et al. (10) to be dependent on calcium in the medium. The apparent calcium-independence of the enzymatic activity sug-

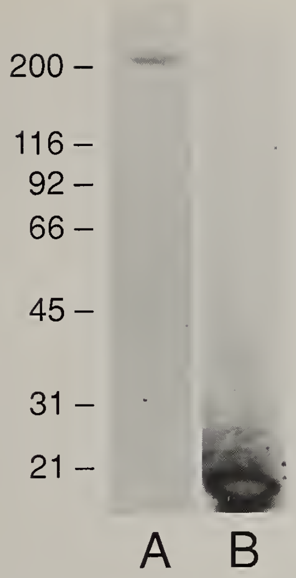

FIG. 1. AC Toxin Binds ^{45}Ca. Adenylate cyclase toxin (16 μg, 74 pmol), lane A, and calmodulin (5 μg, 300 pmol), lane B, were separated on a 5 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis; transferred to polyvinyldene difluoride membrane; probed with 1 μCi/ml of ^{45}Ca; and exposed to X-ray film.

gests that calcium is involved primarily in the interaction of AC toxin with the target membrane and/or its subsequent insertion into or translocation across that membrane.

One approach to dissecting the role of calcium in the intoxication process is to use antagonists. Nickel (Ni) is recognized to block the entry of calcium into eukaryotic cells at the level of calcium channels (23). As shown in Table 2, Ni has only a small effect on the enzymatic activity of AC toxin activated by calmodulin. When added to target Jurkat cells simultaneously with AC toxin, however, Ni elicits a >99% inhibition of AC toxin-induced cAMP accumulation. Not surprisingly, the intoxication of sheep erythrocytes by AC toxin was also inhibited by Ni. In contrast, the ability of AC toxin to cause hemolysis was unaffected by Ni. These data indicate that the initial interaction of AC toxin with erythrocytes (and perhaps other target cells) may be unaffected by Ni, but that some part of the process required for delivery of the enzymatic domain to its intracellular site of catalytic activity is impaired.

Because of the limitations of using inhibitors such as Ni, the direct interaction of calcium with AC toxin and the consequences thereof were investigated. The calcium dependence of toxin and hemolytic activities and the presence of putative calcium-binding domains by DNA sequence analysis of the toxin structural gene raised the possibility that AC toxin might be a calcium-binding protein. The data shown in Fig. 1 confirm that hypothesis. The 216-kDa band which is AC holotoxin (Fig. 1, lane A) binds ^{45}Ca. The calcium-dependent regulatory protein, calmodulin (Fig. 1, lane B), was used as a positive control for the documentation of calcium binding. These data are consistent with the calcium-binding activity of *E. coli* hemolysin observed elsewhere (D. F. Boehm and I. S. Snyder, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B-61, p. 40) and support the hypothesis that the region of homology between the two toxins which contains the GGDGDDTLX repeats is likely to be the mediator of that function.

To determine the consequence of calcium binding to AC toxin, fluorescence spectroscopy was used for detection of intrinsic tryptophan fluorescence. As shown in Fig. 2, the fluorescence emission spectrum of AC toxin exhibits a blue shift in the peak of maximum fluorescence intensity as well as a small change in the spectral profile when changed from 36 nM (solid line) to 1.0 mM (dashed line) free-calcium. These observations suggest that the molecule may be undergoing a conformational change in response to the interaction with calcium. The magnitude of the change is small, but that may reflect modification of the environment of only one or a few of the 16 tryptophan residues present in the toxin.

Visual evidence of a change in AC toxin structure is provided by an ultrastructural electron microscopic study. As demonstrated in Fig. 3 (panel A), AC toxin in

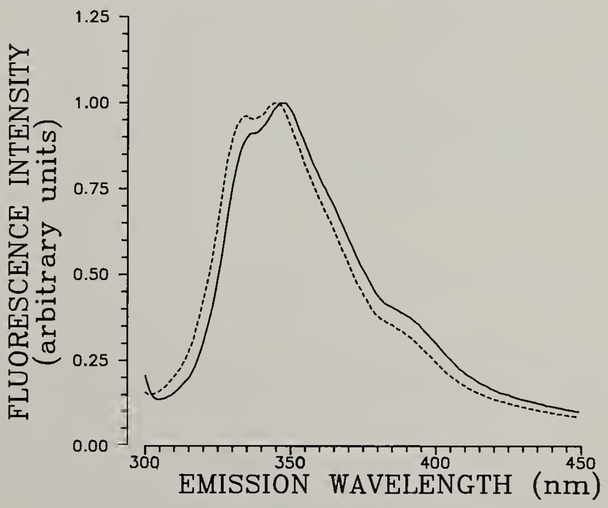

FIG. 2. Fluorescence emission spectra of AC toxin. Intrinsic fluorescence was measured as described in text. Shown are AC toxin (70 μg/ml) in 36 nM Ca^{2+} (——) and 1.0 mM Ca^{2+} (---).

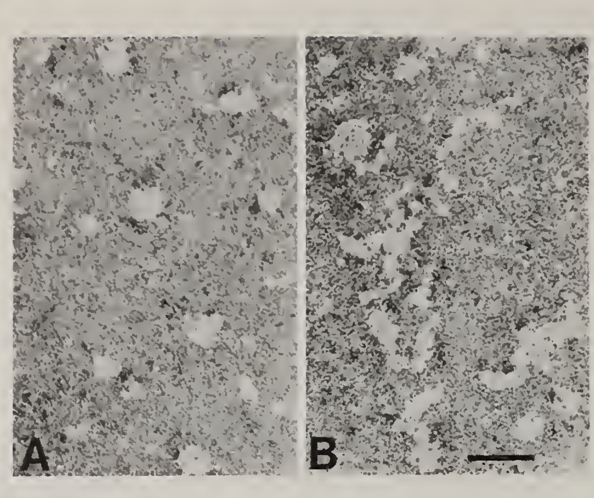

FIG. 3. Electron microscopy of AC toxin. Affinity purified AC toxin was prepared as described in text. Panel A, AC toxin in 36 nM of Ca^{2+}; panel B, 1 mM of Ca^{2+}. Photomicrographs were made at a magnification of 250,000.

36 nM free-calcium (the concentration that does not support toxin or hemolytic activities) appears as a closed globular structure. It is intriguing that maximal enzyme activity can be elicited under these low calcium conditions with the addition of calcium-free calmodulin. When the ambient free-calcium concentration is increased to 1.0 mM, the AC toxin appears to open, assuming a semicircular conformation with some molecules appearing to interact at one end. These ultrastructural data, taken with the results of functional studies, suggest that the open structure elicited by interaction with calcium enables the holotoxin molecule to interact productively with target cells. Additional studies are required to determine the mechanism by which such a structure inserts into the target cell membrane to create a pore leading to hemolysis.

DISCUSSION

Several investigators have observed effects of calcium on the physical properties and/or biological activity of AC toxin (7, 12, 18). In the original description of AC enzyme activity from *B. pertussis*, there was no calcium requirement noted, and calcium could not substitute for magnesium as a substrate cofactor (14). Kilhoffer et al. (15) and Greenlee et al. (11) have shown that the enzymatic activity of AC from *B. pertussis* can be activated by calmodulin in the absence of calcium. The potency of calmodulin in activating the enzyme, however, was increased by calcium (15). Whether this calcium effect is on the AC enzyme or on calmodulin with a secondary effect on its interaction with AC is not clear. Nevertheless, it is clear that AC toxin can express full enzyme activity without calcium.

From the first demonstration of AC toxin activity in urea extracts of *B. pertussis*, Confer et al. noted that the phenomenon required calcium (5). Hanski and Farfel observed that the ability of AC toxin to increase cAMP levels in lymphocytes was calcium concentration-dependent (12). Their data do, however, indicate some toxin activity at what is reported to be a calcium concentration of zero. Hanski and Farfel also observed a shift in the apparent size of the gel chromatography fraction possessing AC toxin activity, from 190 kDa in the presence of calcium (1 mM) to 340 kDa without calcium (2 mM EGTA). More recently, Masure et al. (18) described the properties of several fractions from *B. pertussis* obtained by QAE-Sephadex chromatography. The fraction (Pk 1) containing AC toxin activity (but not one which had AC enzyme activity only) exhibited a calcium-dependent change in Stokes radius from 33.8 ± 0.94 in EGTA (2 mM) to 43.5 ± 1.3 in $CaCl_2$ (2 mM). These authors also noted the calcium dependence of toxin activity and observed a calcium-dependent shift in mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Although they did not address the issue of calcium-dependent shift in size, Gentile et al. (7) noted two forms of AC toxin by analytical centrifugation and demonstrated a shift in intrinsic fluorescence upon denaturation with 6 M guanidine.

The calcium requirement for toxin activity demonstrated in the present work is consistent with data discussed above. That hemolysis elicited by AC toxin is also dependent on calcium is consistent with a calcium-induced change in the molecule being necessary for binding and/or insertion of the toxin molecule. The ability of Ni to dissociate toxin and hemolytic activities raises intriguing questions about the mechanisms of those two functions. While Ni has been shown to block calcium channels in eukaryotic cells (23), it is unclear whether its effect on AC toxin activity occurs by altering the toxin or the target cell. However, the Ni-mediated reduction in toxin activity without a change in hemolytic activity indicates that, although mediated by the same holotoxin molecule, the pathways leading to hemolysis and intoxication must diverge at some point.

The data from Hanski and Farfel (12) and Masure et al. (18) appear to be in conflict regarding the effect of calcium on the apparent size of the AC molecule possessing toxin activity. The data presented here, with a shift in intrinsic fluorescence and an apparent opening of the molecule by calcium, are more consistent with the increase in Stokes radius observed by Masure et al. (18) upon addition of calcium. Adding of calcium to the material (which is purified in the absence of calcium) during sucrose gradient centrifugation resulted in loss of the narrow band of AC toxin activity routinely observed and dispersion of AC toxin-containing material through the gradient (data not shown). This observation suggests a heterogeneity of density in the individual molecules or aggregates that develop as a result of interaction with calcium.

That each of the activities of AC toxin on target cells has an absolute requirement for calcium suggests that the binding and entry process may be dependent upon the calcium-induced conformation. Additional studies are needed to determine which domains of AC toxin recognized in the amino acid sequence of Glaser et al. (8) correspond to the structure now visualized on electron microscopy. These studies will be carried out using monoclonal antibodies, labeled calmodulin, and other reagents. Examination of mutated AC toxin molecules (E. M. Barry, A. A. Weiss, I. E. Ehrmann, M. C. Gray, E. L. Hewlett, M. S. Goodwin, submitted for publication) by these same approaches will provide additional information on the structure/function relationships.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI18000 to (E. L. H.) and DK28942 (University of Virginia Diabetes Center).

The authors thank Elizabeth Robinson, Kathy May, Donna Taylor, Margaret McLeod, and George Vandenhoff for their assistance with this work.

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DISCUSSION

DR. HANSKI: Would you expect that the native molecule will migrate and be bigger in the presence of calcium?

DR. HEWLETT: Obviously, I left unresolved the question of whether it gets larger or smaller. We have not done that specific experiment in a definitive way so I cannot answer that. I would speculate that by gel chromatography, this open molecule would in likelihood increase in apparent molecular size rather than decrease.

DR. LEININGER: From your sequence homology data, do you have any insight where the calcium binding site is on the protein?

DR. HEWLETT: Those repeating motifs which are present in the carboxyl, half of the molecule if you will, there are 43 of those and it is clear to me, even though we do not know the stoichiometry, that we are not binding 43 calciums by each adenylate cyclase toxin. So it is not clear what contribution each of those repeats makes, if any, to calcium binding. That whole area is open for speculation but there are no definitive data to say that they are, in fact, involved in calcium binding. That is really on the basis of homology. That is an important issue that needs to be addressed.

DR. ULLMANN: It is almost the same question. I just wanted to ask you whether you have some truncated (inaudible) in the carboxyl terminus to see whether they bind calcium or not? The other question is whether the secreted material binds the same way?

DR. HEWLETT: Well, in answer to your first question, we do not have any mutants in which we have addressed that issue. We have the mutant that Eileen Barry will talk about which is not activated by the C gene and that binds calcium, apparently in an equivalent manner to the wild type toxin. We have not looked at any of the other mutants that have deletions to determine whether they bind calcium or not. We have looked only with this prepared material which has toxin activity, I cannot answer your question because even with BSA we only get a very small amount of holotoxin accumulating in the culture medium. So I do not have enough of that material to be able to evaluate whether it behaves any differently with regard to calcium binding.

DR. MASURE: Did you assay enzyme activity in the absence of calmodulin plus and minus calcium? The second question is, are there any structural changes plus and minus calcium in the presence of calmodulin?

DR. HEWLETT: Those are good questions. We did not repeat the calcium effect in the absence of calmodulin but the data would suggest that at lower calmodulin concentrations, calcium does have an effect. With no calmodulin at all, I cannot tell you because we did not do that. But calcium increases the potency of calmodulin, as you know from data that Jan Wolff has.

DR. MASURE: But that is at a very low level, right?

DR. HEWLETT: Low level of which?

DR. MASURE: Of either. At a very low level of calcium, that curve is in the nanomolar range if I remember correctly.

DR. HEWLETT: Maybe Jan can comment on that. I did not look at these effects on enzymatic activity in the absence of calmodulin so I did not do that part and I cannot tell you. The other question you asked is a very important one. What does calmodulin do to these conformational changes? Does it interact given the fact that calmodulin in certain cell types can alter the toxin activity? That is something that we are planning to do but I do not have an answer for you.

DR. WOLFF: We and others invariably found that at high concentrations, exceeding 100 micromolar of calcium, catalytic activity is inhibited, and I wonder if that inhibition which you did not show on your slide, can be correlated with the structural change which happens at that plateau level.

DR. HEWLETT: That is a very interesting point. Because it is a log scale, there is a slight downturn there which, if it were plotted on a linear scale rather than a log scale, might have been as much as a 50 percent inhibition, even up to one millimolar which is similar to what you all demonstrated recently with the enzymatic activity. That certainly is possible but given the magnitude of changes that we were seeing otherwise, it was not a major one. It certainly could be related to the confirmational change that occurs.

DR. GUPTA: What are the chances of this toxin being used as a component of the acellular vaccine with regard to detoxification and immunogenicity?

DR. HEWLETT: Those are excellent questions that we are interested in. We are involved in some collaborative studies with Roberta Shahin and Drusilla Burns addressing some of those questions, some of which will be talked about later in this symposium with regard to the immunogenicity of the molecule in people that have had clinical pertussis. At the moment I would say that the availability of the molecule as Dr. Manclark alluded to in a publication from several years ago, one of the critical features is the availability of large quantities of the material to enable it to be tested and to be used in a vaccine. At the moment the clean material that we can obtain is fairly hard to come by. We are very interested in whether or not it might be an important component. I believe there is also a presentation from the people at the Institut Pasteur with regard to the protective activity of the molecule later on in the program.

DR. GUPTA: (Inaudible) ...have you shown the effect of the epitopes on the --

DR. HEWLETT: The majority of the monoclonals that we have are not directed against a calcium dependent epitope so it is only that one. It is at physiologic concentrations of calcium that the antibody does react when the molecule is open so if anything, under those circumstances, I would expect it to be more immunogenic rather than less.

DR. COOKSON: You mentioned the hemolytic ability of purified toxin. As you well know, I am sure that you can get hemolysis in specific and non-specific ways and so I was curious about the concentration of purified material that allows you to detect hemolysis.

DR. HEWLETT: I think the hemolytic activity that Ingrid Ehrmann has demonstrated is specific hemolysis that occurs at concentrations of toxin below one microgram per milliliter and it appears to be dependent on the creation of a pore in the membrane. Osmotic protection experiments show that one can protect with large size sugars such as sucrose and arabinose but not smaller sugars indicating that there may be a pore created in the membrane that is responsible for the hemolytic activity, much in a fashion analogous to that with the *E. coli* hemolysin except that the pore appears to be substantially smaller.

Hemolysis and Uncontrolled Intracellular cAMP Generation are Independent, Separable Functions of the Adenylate Cyclase Toxin from *Bordetella pertussis*

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Bordetella pertussis, the causative agent of whooping cough, produces several virulence factors which have been implicated in the pathogenesis of the disease. Among these factors are hemolysin (Hly) and adenylate cyclase (AC) (24). The latter invades eukaryotic cells and generates, upon activation by host calmodulin (CaM), uncontrolled levels of cAMP (3, 10).

Previous studies, using transposon mutagenesis in *B. pertussis*, suggested close proximity between the genes coding for AC (*cya*) and for Hly (*hly*) (25). Recently, it has been reported that both AC and Hly activities could be restored in *B. pertussis* 348 insertion mutant (AC$^-$, Hly$^-$) by complementation with plasmids carrying the complete *cya* operon (2, 9). This operon is composed of four contiguously arranged genes: *cyaA*, *cyaB*, *cyaD*, and *cyaE* (9). The products of the last three genes were found to be essential for the secretion of the structural gene product (*cyaA*) (9). Synthesis of active *Escherichia coli* α-Hly requires two genes *hlyC* and *hlyA* (11, 18). Two additional genes, *hlyB* and *hlyD*, determine a specific transport system for the HlyA protein (16, 23). The predicted amino acid sequences for *cyaB* and *cyaD* are highly similar to the sequences predicted for *hly*B and *hly*D (9). Furthermore, the amino acid sequence predicted for *cyaA* showed a significant degree of homology with other pore-forming toxins (9). A complementation study of *B. pertussis* 348 insertion mutant showed that a domain on AC was essential in producing a phenotypically hemolytic recombinant strain (9). The domain, which is between residues 640 and 910, displays a particularly high amino acid sequence homology with the internal part of α-Hly. Taken together, these findings led Glaser et al. (9) to propose that the *cyaA* codes for a single polypeptide carrying both CaM-dependent AC and Hly activities; the researchers therefore termed the *cyaA* product "cyclolysin" (9).

This study demonstrates that the toxic form of *B. pertussis* AC, i.e., the form capable of penetrating eukaryotic cells and generating cAMP, also possesses a hemolytic activity. The hemolysis mediated by AC toxin occurred after a lag time of 40 to 80 min and required toxin activities higher than those needed for cAMP generation. In addition, cAMP generation was absolutely dependent on extracellular Ca^{2+}, whereas hemolysis occurred in the absence of Ca^{2+} and the presence of EGTA. Furthermore, cAMP production in response to the toxin was blocked by exogenous CaM, whereas hemolysis was enhanced. AC toxin induced cAMP accumulation in human erythrocytes but did not cause hemolysis. These data together suggest that the toxic and hemolytic functions of *B. pertussis* AC toxin are separable and probably mediated by different domains on the AC toxin polypeptide.

MATERIALS AND METHODS

ATP, cAMP, 8-Br-cAMP, bovine brain CaM-agarose, protein A, protein standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), bovine serum albumin (BSA), and EGTA were from Sigma Chemical Co., St. Louis, Mo. Ficoll-Hypaque and Ultrogel AcA34 were from Pharmacia LKB Biotechnology Inc. The researchers obtained [α-^{32}P]ATP (80 Ci/mmol), [^{3}H]cAMP (27 Ci/mmol), and Na^{125}I (1,000 Ci/mmol) from the Radiochemical Centre (Amersham, England). All other reagents used were of the highest purity available.

Culture of organism and purification of AC toxin. The recombinant strain of *B. pertussis* BP348 pRMB1 was constructed as described before (2). This strain was

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* Presenting author.

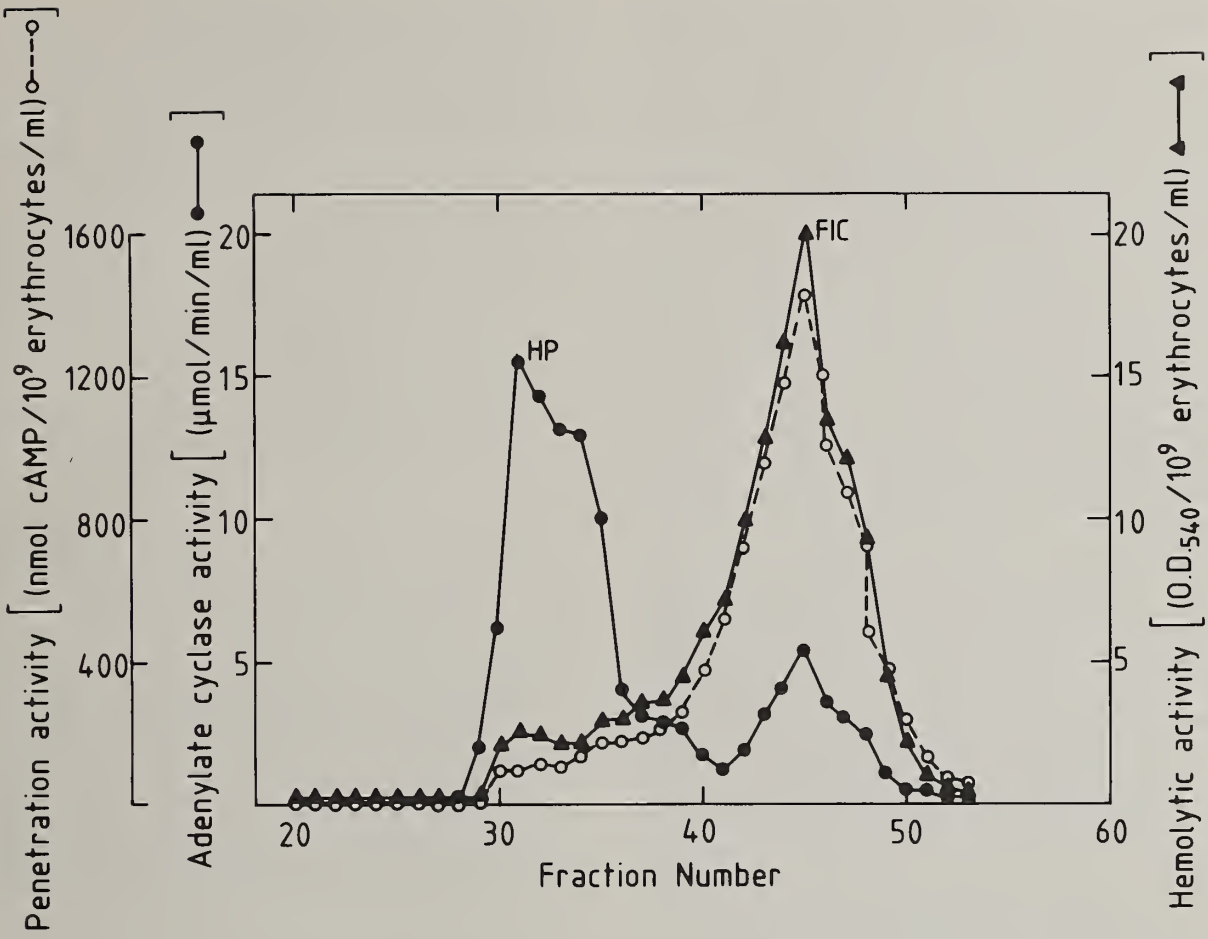

FIG. 1 Isolation of the hemolytically active form of *B. pertussis* AC from bacterial extract. Chromatography of dialyzed urea from the recombinant strain BP348 pRMB1 on Ultragel AcA34 column was performed as described in text. Fractions were assayed for the AC enzymatic activity after 1:200 dilution in TME buffer. To determine the toxic and hemolytic activities, 20 µl from each fraction was incubated with a 1-ml suspension of sheep erythrocytes (5 x 10^8 cells) at 36°C. After 40 min and 3 h of incubation, the amount of intracellular cAMP and the extent of hemolysis were determined, respectively.

passaged twice on Bordet-Gengou agar plates, then inoculated into modified Stainer-Scholte liquid medium and grown for 16 to 20 h in the presence of 10 g/ml of tetracycline and 25 µg/ml of kanamycin to an OD$_{650}$ of 0.6 to 0.8. Bacterial cells were extracted with 4 M urea, and the extract was subjected to gel filtration on an Ultrogel AcA34 column which was equilibrated and run as detailed previously (19, 20). The resolved peak of AC activity containing the toxic form of the enzyme (FIC, see Fig. 1) was concentrated ten-fold on Amicon PM30 membrane and kept at -80°C for further use. The enzyme had a specific activity of 10 to 30 mol/min per mg and a protein concentration of 2 to 5 mg/ml. Complete purification of the toxic form of AC was performed as described before (19).

AC enzymatic activity. The enzymatic activity of AC was measured by quantifying the conversion of [α^{32}P]ATP to [^{32}P]cAMP in a cell-free assay, as described previously (10). Briefly, the reaction was performed in a total volume of 50 µl at 36°C for 15 min. The assay mixture contained 50 mM Tris-HCl pH 7.5, 60 µM CaCl$_2$, 1 mM [α^{32}P]ATP (specific activity $\propto$ 40 cpm/pmol), 10 mM MgCl$_2$, and 10 µM CaM. The [^{32}P]cAMP formed was isolated according to the procedure of Salomon et al. (21). All results are means of duplicate or triplicate determinations which differed by less than 7%.

AC toxin activity. The AC toxin activity was determined by quantitation of intracellular cAMP accumulation in erythrocytes exposed to the toxin, as described before (10, 19, 20). Briefly, erythrocytes (5 x 10^8 cells) were incubated with AC toxin at 36°C. At the end of the incubation, the cells were centrifuged for 2 min in an Eppendorf centrifuge and cell-free supernatants removed by aspiration. Intracellular cAMP was extracted by adding 0.5 ml of a 50 mM sodium acetate solution (pH 4.2) containing 0.1 mM IBMX; the cells were then boiled for 5 min and centrifuged for 15 min in an Eppendorf centrifuge. The cAMP content was quantified by the binding assay devised by Gilman (8). All results are means of duplicate determinations which differed by less than 10%.

Determination of hemolytic activity. Hemolytic activity was assessed by incubating erythrocyte suspensions containing 5 x 10^8 cells with preparations of AC toxin in a Tris-buffered saline (TBS) solution containing 145 mM NaCl, 5 mM glucose, 2 mM CaCl$_2$, 10 mM Tris-HCl pH 7.4 and supplemented with 1 mg/ml of BSA. At the end of the incubation, unlysed erythrocytes were pelleted by centrifugation at 12,000 x g for 2 min, the supernatant fluids were decanted, and the OD$_{540}$ was read using as a reference a TBS solution containing the AC toxin but no erythrocytes. The OD$_{540}$ values corresponding to complete hemolysis were obtained by lysing the erythrocytes with Triton X-100. Spontaneous hemoglobin release was determined by incubating erythrocyte suspensions, under identical conditions, in the absence of AC toxin. The extent of spontaneous hemolysis was subtracted from the results shown and did not exceed 2% of the total hemolysis. All results are means of duplicate determinations which differed by less than 12%.

Preparation of erythrocytes and toxin-treated erythrocyte membranes. Whole fresh heparinized blood of either sheep, rabbits, mice, or humans was centrifuged at 400 x g for 10 min. After removing the upper layer containing the plasma and buffy coat by aspiration, the erythrocytes were washed twice in a TBS solution as described above. Erythrocytes were then resuspended in the TBS solution and centrifuged at 400 x g through a Ficoll-Hypaque gradient for 40 min at 36°C. The gradient's upper layer was removed by aspiration and, prior to use, the erythrocytes were washed twice in TBS solution by repeated centrifugation as described above. This procedure allowed the erythrocytes to separate almost completely from other blood constituents. Erythrocyte suspensions (5 x 10^8 cells) were incubated with AC toxin at 36°C in TBS solution. Either partially or completely lysed cells were pelleted by centrifugation at 12,000 x g for 5 min at 4°C, and the supernatant fluids were decanted and used to determine the AC enzymatic activity and extent of hemolysis. The pelleted cells and cell ghosts were completely lysed by adding 1 ml of a solution containing 10 mM Tris-HCl pH 7.4, 2 mM MgCl$_2$, and 1 mM EDTA (TME); they were then frozen and thawed twice using liquid nitrogen. Erythrocyte membranes were washed twice with TME by repeated centrifugations at 12,000 x g for 15 min, and then finally resuspended in 100 1 of TME. Two 10 µl samples were withdrawn for AC enzymatic activity determination; the rest of the sample was used for SDS-PAGE.

SDS-PAGE, immunoblotting, and iodination. SDS-PAGE was performed according to Laemmli (14) on 1.5-mm thick SDS-polyacrylamide gels. Proteins were separated on 9% SDS-PAGE for immunoblotting, transferred to nitrocellulose sheets, and incubated with polyclonal anti-AC antibodies; the corresponding interacting bands were visualized with ^{125}I-labeled protein A as previously described (20). AC iodination was conducted essentially according to Ladant's procedure (13). Before eluting the enzyme from CaM-agarose column, 70 µl of packed resin was withdrawn and used for iodination. The iodinated enzyme was eluted with 8.8 M urea, passed through a G-25 column equilibrated and run in a solution of 50 mM Tris-HCl pH 7.5, 0.1 mM CaCl$_2$, and 0.1 mg/ml of BSA. The activity of the iodinated enzyme was 5 to 10 µmol/min per 10^7 cpm of ^{125}I.

RESULTS

The toxic form of *B. pertussis* is hemolytically active. The AC activity of *B. pertussis* has been associated with several polypeptides of different molecular mass ranging from 43,000 to over 660,000 kDa (17). Recently, it has been shown that AC is synthesized as a 200-kDa polypeptide which is the product of the *cyaA* gene (12, 19, 20). The apparent SDS gel molecular mass of ∝ 200 kDa is anomalous and the value obtained by equilibrium centrifugation is 176 kDa, which is in good agreement with the value deduced from *cyaA* sequence [6].) The heterogeneity of the various AC forms results from proteolytic degradation and molecular aggregation of the 200-kDa form of the enzyme (19, 20). Recently, Rogel et al. isolated two enzymatically active forms of AC, both 200 kDa, from a recombinant *B. pertussis* strain (19). Only one of these was toxic, i.e., capable of gener-

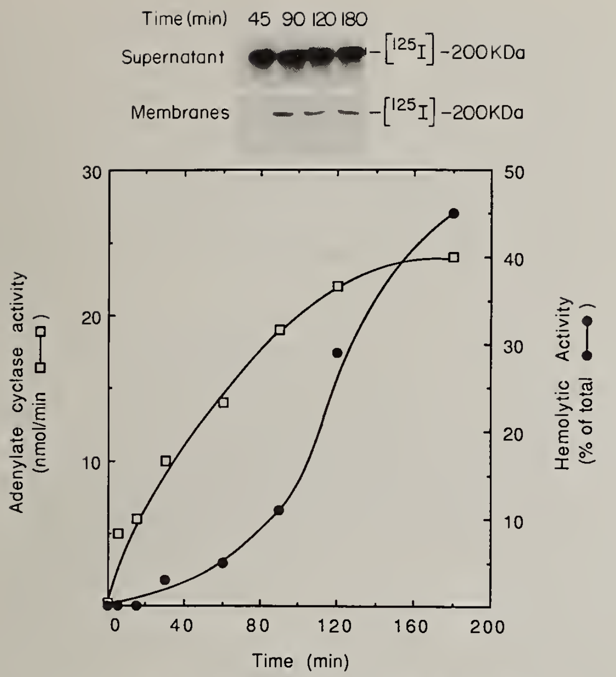

FIG. 2. The purified AC toxin is hemolytically active. The AC toxin was purified by CaM-agarose and iodinated as described in text. The ^{125}I-labeled AC toxin (1 μmol/min, 2 x 10^6 cpm) was incubated with a suspension of sheep erythrocytes (5 x 10^8 cells) in a TBS solution. At the indicated times, the extent of hemolysis and the amount of enzymatic AC activity bound to erythrocyte membranes were determined. The upper panel shows the autoradiography of SDS-PAGE containing samples of cell-free medium and membranes.

ating intracellular cAMP in eukaryotic target cells (19). Figure 1 demonstrates that a similar resolution between the toxic and nontoxic forms of AC was obtained when sheep erythrocytes were used as target cells. The aggregated peak of the enzymatic activity (HP) contained only a small amount of the toxic activity, whereas the toxic activity migrated as a distinct peak (FIC) with apparent size of about 200,000 Daltons. When the same fractions were incubated with sheep erythrocytes for 3 h, hemolysis developed. The distribution of the hemolytic activity completely coincided with that of AC toxic activity (Fig. 1). To provide unequivocal evidence that the AC toxin possesses hemolytic activity and to identify the membrane-bound form of AC toxin, the researchers purified it to homogeneity, iodinated it, and incubated it with sheep erythrocytes. Fig. 2 demonstrates that CaM-dependent AC activity accumulated gradually in the plasma membranes of sheep erythrocytes. At the same time, there was no significant decrease in the enzymatic activity or radioactivity of AC toxin in the incubation medium (not shown). The amount of AC enzymatic activity, and of radioactivity that became attached to the erythrocyte plasma membranes, after 3 h of incubation constituted only 2.4% and 1.5%, respectively, of the total AC activity and radioactivity applied. Hemolysis of erythrocytes could be detected after a lag time of about 60 min; after 3 h of incubation, 45% of the cells were lysed. SDS-PAGE and autoradiography of the corresponding erythrocyte membranes revealed the presence of only the 200-kDa form of *B. pertussis* AC. No other lower-mass forms of the enzyme were detected by autoradiography using iodinated toxin or by immunoblotting of membranes using anti-*B. pertussis* AC antibodies. The potency of purified AC toxin to induce cAMP accumulation and to lyse sheep erythrocytes varied considerably in different preparations, although all contained only the 200-kDa form of the enzyne. FIC, the toxic form of AC which was resolved by gel filtration from bacterial extract (Fig. 1), had steadier activities and produced results similar to those presented in Fig. 2. It was therefore used as a source for AC toxin in this study.

The relationship between cAMP accumulation and hemolysis. The time course of cAMP accumulation and hemolysis development in sheep erythrocytes exposed to AC toxin is shown in Fig. 3. Clearly, cAMP accumulation preceded erythrocyte lysis; the accumulation was

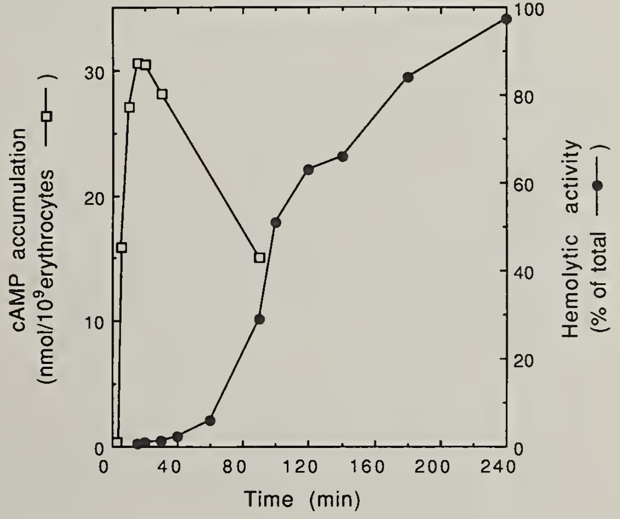

FIG. 3. Time courses of cAMP generation and hemolysis in sheep erythrocytes. FIC (0.15 μmol/min) was incubated with sheep erythrocytes. At the indicated times, the amounts of intracellular cAMP and the extent of hemolysis were determined.

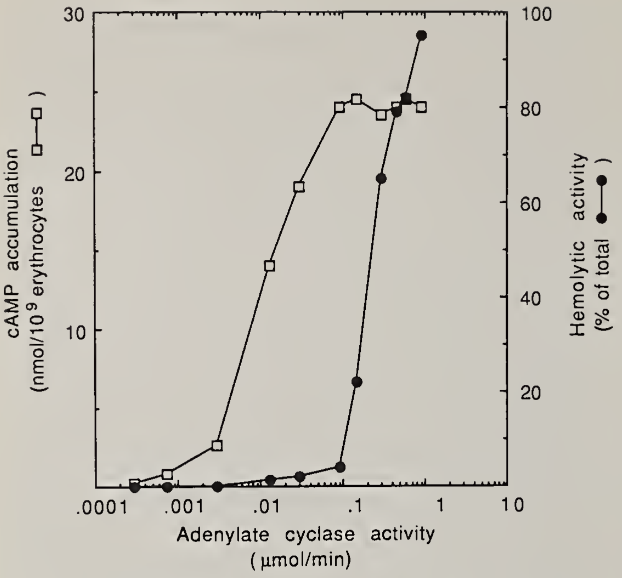

FIG. 4. Dose-dependencies of cAMP generation and hemolysis on AC toxin. Sheep erythrocytes were incubated with the indicated activities of FIC. Intracellular cAMP and the extent of hemolysis were determined after 30 min and 3 h of incubation, respectively.

immediate and reached maximal levels within 20 to 40 min. In contrast, hemolysis began to be apparent only after a lag time of about 1 h and reached a maximal level within 4 h of incubation (Fig. 3). The dose-dependencies of cAMP accumulation and of hemolysis on AC toxin are shown in Fig. 4. AC toxin, at activities of 0.01 to 0.05 µmol/min, generated an almost maximal level of intracellular cAMP without causing a significant hemolysis. The half-maximal concentration of AC toxin required for cell lysis was at least 10 times higher than that required for cAMP generation (Fig. 4).

Experiments showed that extracellular Ca^{2+} at mM concentrations was absolutely required for cAMP generation induced by AC toxin, but not for hemolysis. Hemolysis occured in the absence of extracellular Ca^{2+} and the presence of EGTA, but was further delayed as compared to its appearance in the presence of Ca^{2+}. The experiments shown in Fig. 5 were conducted to test whether the binding of AC toxin to erythrocytes in the absence of Ca^{2+} was functional in the sense that it could induce cAMP when Ca^{2+} was replenished in the solution bathing the cells. Sheep erythrocytes were incubated for 30 min with AC toxin in the absence of Ca^{2+} and the presence of EGTA. The cells were then washed and reincubated in toxin-free solutions containing either Ca^{2+} or EGTA. As shown in Fig. 5, the reincubation of toxin-treated cells in the presence of Ca^{2+} led to a rapid cAMP accumulation; no cAMP was produced in cell reincubation in the presence of EGTA. However, hemol-

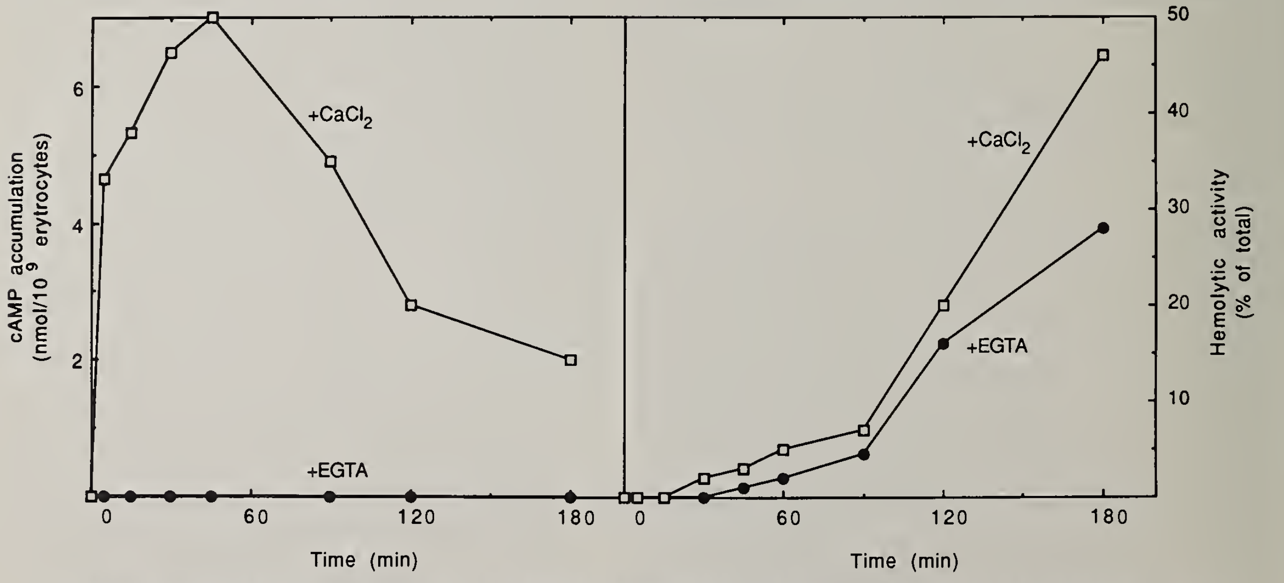

FIG. 5. Binding of AC toxin to erythrocytes in the presence of EGTA leads to accumulation of cAMP when Ca^{2+} is replenished in the cell medium. Sheep erythrocytes were incubated with FIC (1.2 µmol/min) for 30 min at 36°C in a TBS solution containing 2 mM EGTA and no $CaCl_2$. Cells were then washed twice with a cold TBS solution containing 0.1 mM EGTA (and no $CaCl_2$) and divided into two groups. One group was reincubated in a TBS solution containing 2 mM $CaCl_2$ (□ - □); the second group of cells was reincubated in a TBS solution containing 2 mM EGTA and no $CaCl_2$ (●-●). At the indicated times, intracellular cAMP (left panel) and the extent of hemolysis (right panel) were determined.

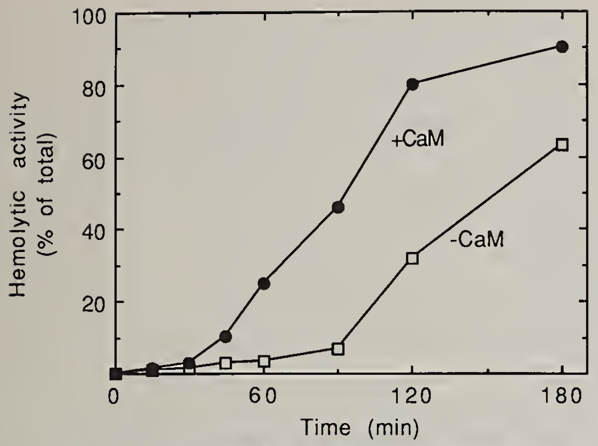

FIG. 6. Exogenous CaM potentiates the hemolytic response of sheep erythrocytes to AC toxin. FIC (0.15 µmol/ml) was incubated in the presence or absence of 5×10^{-5}M CaM for 1 h at 4°C. Sheep erythrocytes were then added and the extent of hemolysis determined at the indicated times.

ysis developed in erythrocytes incubated in the presence of either Ca^{2+} or EGTA. The extent of lysis after 3 h of cell reincubation in the presence of EGTA was lower than that in cells reincubated in the presence of Ca^{2+} (Fig. 5).

Exogenous CaM at mM concentrations has been shown to inhibit cells' cAMP response to AC toxin in its partially purified (22) and completely purified (19) forms. The researchers tested the effect of exogenous CaM on the hemolytic activity mediated by AC toxin. As shown in Fig. 6, exogenous CaM potentiated the hemolytic activity of AC toxin; the lag time in the appearance of hemolysis was shortened, and the extent of lysis was considerably increased. Under similar conditions, CaM almost completely blocked the cAMP generation (not shown).

While testing the susceptibility of erythrocytes from various mammalian sources to AC toxin, the researchers found that human erythrocytes produced cAMP in re-

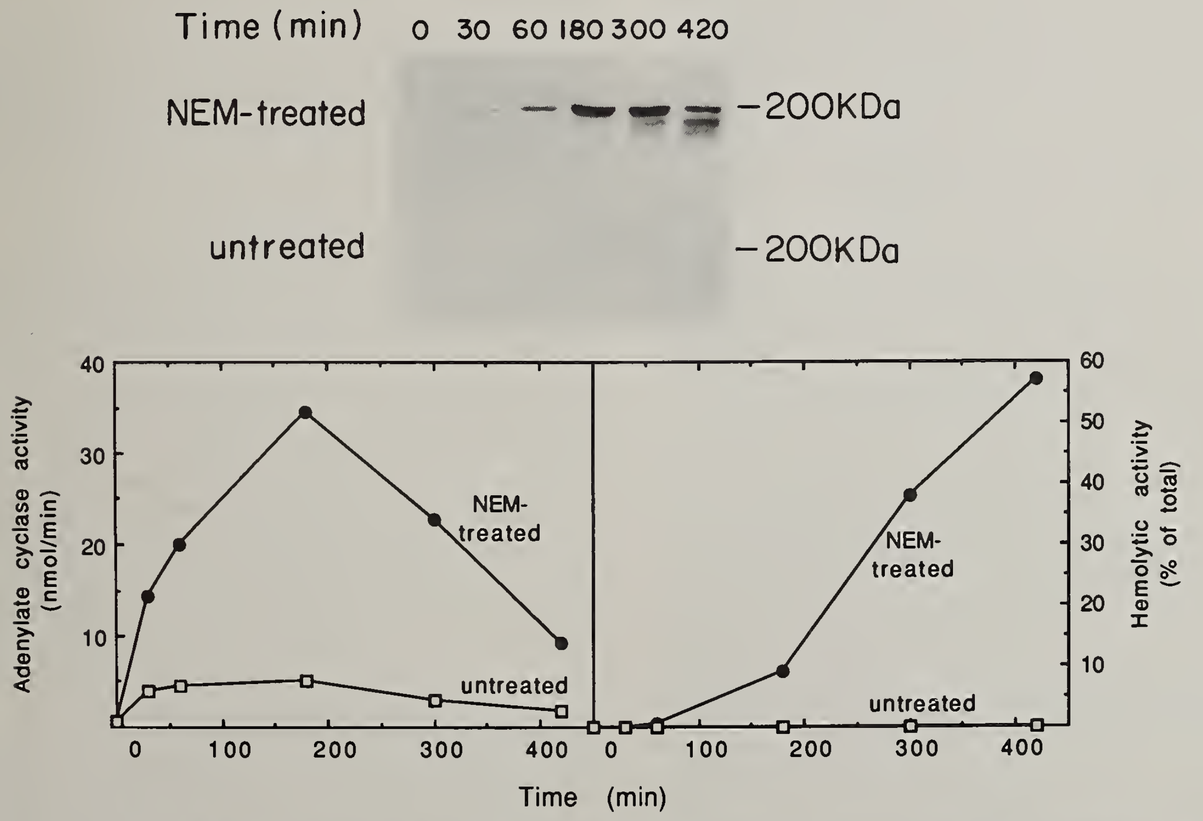

FIG. 7(A). Human erythrocytes are resistant to the hemolytic activity of AC toxin but become susceptible upon NEM-treatment. Human erythrocytes (5×10^8 cells/ml) were incubated with 1 mM NEM in a TBS solution (without BSA) for 10 min at 36°C. Glutathione (reduced) was then added in a final concentration of 2 mM, and cells were washed twice in cold TBS solution. NEM-treated (●-●) or untreated cells (□-□) were then incubated with FIC (1.2 µmol/min). At the indicated times, the amounts of AC enzymatic activity bound to erythrocyte membranes (left panel) and the extent of hemolysis (right panel) were determined. Immunoblotting of membranes from human erythrocytes, either NEM-treated or untreated, using polyclonal anti-*B. pertussis* AC antibodies, are shown in the upper panel.

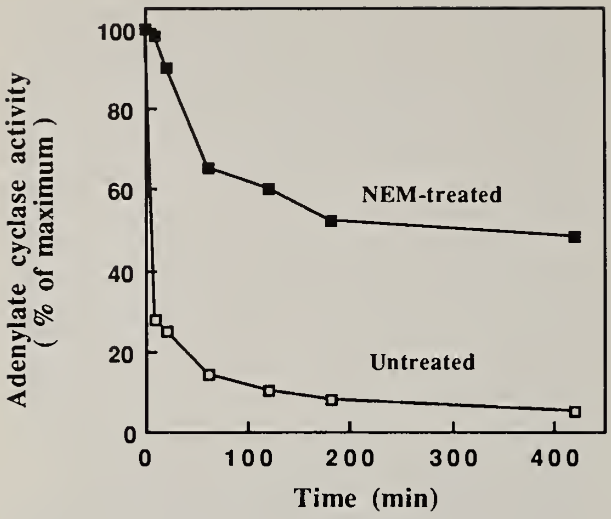

FIG. 7 (B). The decay of AC activity bound to human erythrocytes. Human erythrocytes were treated with NEM as described in (A). NEM-treated and untreated erythrocytes were incubated with FIC (1.2 µmol/min) for 30 min at 36°C. Cells were then washed twice and reincubated in a toxin-free TBS solution. At the indicated times, the amount of AC enzymatic activity bound to erythrocyte membranes was determined. Maximum AC activity (100%) represents activities of 15 µmol/min and 4 µmol/min for NEM-treated and untreated cells, respectively.

Table 1. Susceptibility of erythrocytes of various sources to the hemolytic activity of AC toxin[a]

Source of erythrocytes	Hemolytic activity (% of total)	AC activity (membrane-bound) (nmol/min)	cAMP accumulation (nmol/10^9 cells)
Sheep	98	114	24
Humans	0	4	8
Rabbits	100	126	20
Mice	95	112	16

[a] Sheep, rabbit, human, and mice erythrocytes (5×10^8 cells) were incubated with AC toxin (1.2 µmol/min) at 37°C. The cAMP accumulation was determined after 40 min and the degree of hemolysis and the enzymatic activity of AC toxin bound to erythrocyte membranes were determined after 3 h of incubation as explained in text.

sponse to AC toxin but were completely resistant to lysis. Table 1 shows that the amount of cAMP produced in human erythrocytes was two to three times lower than in erythrocytes from other sources. However, the amount of AC toxin which bound to human erythrocyte membranes was about 30 times lower than that bound to membranes of erythrocytes from other sources. Immunoblot analysis of membranes from human erythrocytes exposed to AC toxin revealed the presence of tiny amounts of 200-kDa AC, which seemed to become degraded at prolonged incubation periods (Fig. 7A). Treating these cells with 1 mM of N-ethylmaleimide (NEM) for 10 min at 36°C prior to their exposure to AC toxin rendered them susceptible to lysis (Fig. 7A). NEM-treated human erythrocytes accumulated much higher levels of AC toxin than untreated cells; this finding was detected by both immunoblotting and by assaying the enzymatic membrane-bound AC activity. The highest amount of the 200-kDa form of AC bound to membranes of NEM-treated erythrocytes was detected after 3 h of cell incubation with AC toxin. Longer incubation resulted in a decrease in the bound AC activity and degradation of the 200-kDa AC to lower molecular weight forms (Fig. 7A). NEM-treated erythrocytes inactivated the cell-bound AC toxin at a slower rate than did untreated cells; this was determined by reincubating toxin-treated erythrocytes in toxin-free buffer (Fig. 7B).

DISCUSSION

Several investigators recently have demonstrated that the 200-kDa AC of *B. pertussis* is able to raise intracellular cAMP levels in mammalian cells when added in its purified form to cell media (6, 12, 19). Based on sequence homology to *E. coli* α-Hly, Glaser et al. (9) predicted that *B. pertussis* AC should possess hemolytic activity. Recently we have isolated two forms of AC from a recombinant *B. pertussis* strain; both of these were 200 kDa, yet only one form was toxic and generated cAMP in target cells (19). The present study showed that the toxic form of *B. pertussis* AC is the only one capable of lysing erythrocytes. Therefore, it is conceivable that the process which activates *B. pertussis* AC and produces the toxic form may also be responsible for the generation of a hemolytically active protein. Since cAMP production and hemolysis seem to be independent functions (see below), the process of AC toxin activation is probably required for the attachment of AC toxin to its target cell.

Hemolysis appeared to be a distinct, temporally dissociated, step in toxin-mediated cAMP production. By manipulating the composition of sheep erythrocyte incubation medium, the researchers could completely dissociate between these two functions of AC toxin.

Exclusion of Ca^{2+} and addition of the chelating agent EGTA did not prevent AC toxin binding and cell lysis but did completely abolish cAMP generation. Ca^{2+} was identified as playing a role in the binding of *E. coli* α-Hly to erythrocytes through 11 tandemly repeated sequences consisting of nine amino acids (15). Glaser et al. identified similar nonomers on *B. pertussis* AC (9). Recently, it was shown that the hemolytic activity of α-Hly produced by *E. coli* grown in the absence of Ca^{2+} was Ca^{2+}-dependent, whereas the α-Hly produced by *E. coli* grown in the presence of Ca^{2+} was Ca^{2+}-independent (1). It is possible that culturing of *B. pertussis* in the absence of Ca^{2+} will produce an AC toxin whose hemolytic activity will also be Ca^{2+}-dependent.

Reincubating erythrocytes pretreated with AC toxin in the presence of EGTA in a toxin-free buffer containing Ca^{2+} resulted in an immediate rise in intracellular cAMP; reincubating these cells in buffer containing EGTA, however, prevented elevation of intracellular cAMP. These results suggest that Ca^{2+} is absolutely required for the insertion of the domains on AC toxin required for cAMP production into the cell cytosol. CaM binding to the AC toxin inhibited the insertion of these domains but enhanced hemolysis. This suggests that changes in the conformation of the N-terminal part of AC may affect those protein domains responsible for hemolytic activity.

Pertussis is a disease of humans; however, human erythrocytes failed to respond to the hemolytic activity of AC toxin. This resistance to lysis may have evolved from the ability of human erythrocytes to inactivate the invading AC toxin (5). Such inactivation was also observed in human lymphocytes (4) and was attributed to proteolytic degradation of AC toxin by a protease found in the host cell (7). It appears that the lifetime of AC toxin in human erythrocyte membranes is too short to allow the formation of an effective lesion but is long enough for substantial amounts of intracellular cAMP to be generated. Human erythrocytes represent a natural occurring system that can discriminate between these two functions of AC toxin.

ACKNOWLEDGMENTS

This work was supported in part by grants from the U.S.A.-Israel Binational Science Foundation, the U.S.A.-Israel Cooperative Development Research Programme, and the Pasteur-Weizmann Joint Research Program to E.H.

We are grateful to Rona Levin for excellent processing of this manuscript, and we thank Zvi Farfel for critical reading of this manuscript.

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DISCUSSION

DR. BRENNAN: I was unclear what the non-toxic form is. Is it an aggregate of the toxic form?

DR. HANSKI: No. The non-toxic form is a molecule of 200,000 which we can purify from an urea extract of the bacteria. It has calmodulin dependent adenylate cyclase activity but it will not produce cyclic AMP in target cells and it will not lyse these target cells. The only difference between the non-toxic form and the toxic form is the migration on gel filtration. As far as I know, there is some genetic evidence emerging now that the basis for this phenomenon may be a (inaudible) modification that converts the non-toxic form to the toxic form similarly as was found for the *E. coli* hemolysing system.

DR. BRENNAN: Were antibodies directed against the non-toxic form able to react with the toxic form?

DR. HANSKI: Yes, we do not have yet antibodies that will distinguish between these forms. Such antibodies were produced recently for the *E. coli* hemolysing.

DR. BRENNAN: But do you have ones that cross-react?

DR. HANSKI: Yes, the antibody that we have cross-reacts with the two forms.

DR. BRENNAN: So I am thinking about cyclase as a possible vaccine candidate. Then the non-toxic form would be a possibility for use as a component.

DR. HANSKI: Well, it might be. I think the only difference between these two forms is this modification which probably is essential for binding of this toxic form to the target cells.

DR. EHRMANN: Have you mapped your antibodies that you showed inhibited hemolytic activity? Was it a polyclone or a monoclone?

DR. HANSKI: They were polyclonal antibodies.

DR. EHRMANN: Do you (inaudible) where it starts?

DR. HANSKI: Not yet.

DR. MASURE: I missed something I think. You showed that with initial intoxication with the adenylate cyclase that there is an increase in cyclic AMP within your cell and that it decreases with time.

DR. HANSKI: It decreases with time due to the lysis of the cells.

DR. MASURE: Fine. I missed that. But it is due to the lysis of the cells?

DR. HANSKI: I am measuring intracellular cyclic AMP so the cells are lysed, so actually there is a --

DR. MASURE: So that is not a real decrease in cyclic AMP.

DR. HANSKI: No, it is just that the cells are lysed.

DR. WOLFF: Regarding the large forms of the active invasive cyclase. At concentrations required for equilibrium centrifugation, you can easily see a large form, probably a tetrimer and that is reversibly brought back to the 177,000 one and then back to the large one depending on the conditions of the solution.

DR. HANSKI: After purification, we were trying to convert these forms one to the other and were not able to convert these forms one to the other. So that is the best work we have done. I do not know, but I think that they are really different.

DR. FRIEDMAN: What concentration of cyclase were you using in these studies?

DR. HANSKI: You could see in the slide that I showed the dose dependent concentration. You can get cyclic AMP generation with 10 nanograms of the cyclase and get lysis with 100 nanograms to 1 microgram of the toxin.

DR. POOLMAN I think you have nicely shown that the human erythrocyte is not the prime target cell for the adenylate cyclase.

DR. HANSKI: Not for the hemolytic activity of the adenylate cyclase but it would still make a substantial amount of cyclic AMP.

DR. POOLMAN Did you do testing for cytolytic activity of immunologically active cells like T and B cells, monocytes?

DR. HANSKI: No, not yet.

Effect of Exogenous Agents on the Action of *Bordetella* Heat-Labile Toxin

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Heat-labile toxin (HLT) is a potent, heat-sensitive toxin that is produced by virulent forms of *Bordetella* spp. HLT is one of only two toxins produced by all species of *Bordetella* (10); the other toxin is tracheal cytotoxin (10, 11). Since all *Bordetella* spp. produce similar diseases (2, 12, 13, 15, 16), albeit in different hosts, the possibility exists that HLT may play a role in the virulence of these organisms.

Although HLT can be lethal when injected intravenously or intraperitoneally into animals (5), the best studied action of this toxin is its ability to induce skin lesions in animals when injected intradermally. Because of this effect, the molecule has also been called dermonecrotic toxin (1, 3, 10, 18). The ability of HLT to produce hemorrhagic skin necrosis depends on both species and age of the animal (14, 17, 20). For example, whereas HLT causes blood vessel contraction in the skin of both suckling mice and guinea pigs (4), it produces hemorrhagic necrosis only in suckling mice: adult mice are refractory to this action of the toxin (20). Crude preparations of the toxin produce hemorrhagic necrosis in guinea pigs, but the lesion produced in rabbits has been described as ischemic-like with or without hemorrhage (14).

This report investigates the mechanisms by which HLT produces these different types of skin lesions. Many early studies of HLT used crude preparations of the toxin which were likely contaminated with endotoxin as well as other proteins. Moreover, these studies did not necessarily take into account the fact that animal skin contains many proteins and lipids which might affect toxic action. The present study examined the possibility that exogenous agents which contaminate crude preparations of HLT or agents found in animal skin might influence the types of lesions produced by HLT. We identified several agents that affect HLT, characterized them, and determined their effects on HLT activity.

MATERIALS AND METHODS

Capric, lauric, and stearic acids; fatty alcohols; neutral lipids, phospholipids; and cholesterol were obtained from Serdary Research Laboratory Inc. (London, Ontario, Canada). Other free fatty acids, prostaglandin $F_{2\alpha}$, leukotriene B_4, and bovine serum albumin (BSA), fraction V, were obtained from Sigma Chemical Co., St Louis, Mo. An *Escherichia coli* endotoxin available in the Pregel kit supplied by Seikagaku-Kogyo Co., Tokyo, Japan, was used. Synthetic lipid A (compound 506) was obtained from Daiichi Pure Chemicals, Tokyo, Japan.

Female Hartley guinea pigs, weighing 300 to 600 g, were obtained from Tokyo Experimental Animal Supply Co., Tokyo, Japan. Female Wistar rats, weighing 150 to 450 g, and female ICR mice, 0.5 to 10 weeks old, were purchased from Charles River Japan Co., Tokyo, Japan.

Purified HLT. HLT was purified from a supernatant extract of *B. parapertussis*, strain 23054, phase-I organisms as previously described (5, 6).

Ischemia-inducing and dermonecrotizing activities. Ischemia-inducing and dermonecrotizing activities of HLT were quantified by using female Hartley guinea pigs weighing 300 to 350 g. Samples (100 µl) of an HLT preparation which had been diluted logarithmically in phosphate-buffered saline (PBS) were injected intracutaneously into the animals' shaved backs.

After 8 h, the diameter of the ischemia was measured. After 24 h, the colored lesions, ranging from light reddish (erythema) to purple (hemorrhagic necrosis), were measured. The minimum quantity of protein producing an

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* Presenting author.

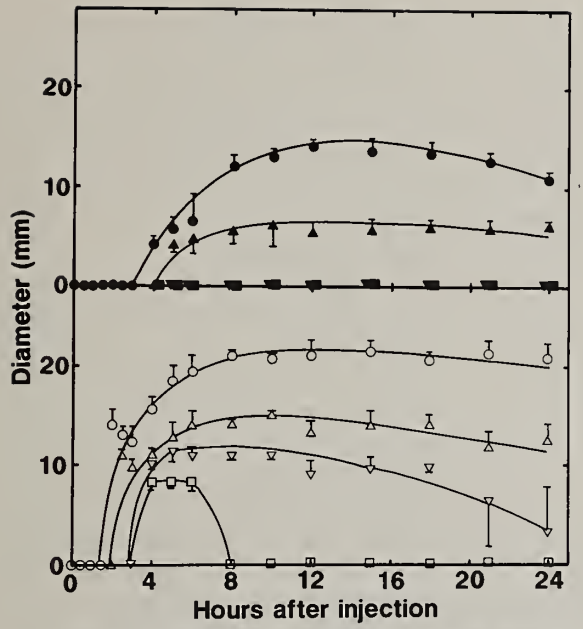

FIG. 1. Time course of HLT action. The diameters of erythemic (closed symbols) and ischemic (open symbols) lesions were measured at the indicated times after intracutaneous injection of 3 (●, ○), 1 (▲, △), 0.3 (▼, ▽), or 0.1 (■, □) MID of purified HLT in a total volume of 0.1 ml. Each symbol with a vertical line represents the mean ± standard deviation of triplicate determinations. Reproduced with permission of *Infection and Immunity* (15).

ischemic lesion 10 mm in diameter was defined as the minimum ischemia-inducing dose (MID). Values reported for MID are means calculated from the dose-response curves for three test animals.

Inhibition of HLT activity. A 200-µl sample in PBS was mixed with HLT (300 MID in 100 µl) in glass tubes. After agitation for several minutes, the solution was diluted logarithmically with PBS and injected intracutaneously into the backs of guinea pigs. At 8 h after injection, the diameter of ischemic lesions was measured.

Assay of endotoxin. Endotoxin was measured by a *Limulus* amebocyte lysate assay, using the Pregel kit from Seikagaku-Kogyo Co. The assay was conducted according to manufacturer instructions.

Methylation of lipids. Etherial diazomethane, prepared by the method described previously (21), was added to the lipid samples (evaporated under nitrogen gas stream) until the color of the samples remained yellow.

Gas chromatography and electron impact mass spectrometry. Samples of methylated preparations were analyzed on a combined gas chromatography-mass spectrometer (JMS DX-300 spectrometer, Jeol Ltd, Tokyo, Japan) with an SBP-5 column (0.2 mm x 20 m; Supelco Inc., Bellefonte, PA). The injector block temperature was maintained at 270°C. The column temperature was first set at 170°C for 2 min and then programmed at 8°C/min. Helium flow rate was 1 ml/min. A flame ionization detector was used to monitor the chromatographic signals.

Statistical methods. Data were tested for statistical significance by Student *t* test.

RESULTS AND DISCUSSION

Effects of exogenous agents on HLT action on guinea pig skin. Crude preparations of HLT have been reported to produce hemorrhagic lesions in guinea pig skin (14, 19). This study found that as HLT is purified, its ability to produce hemorrhagic lesions decreased. The purified toxin, however, did produce ischemic lesions in the skin of guinea pigs (6), rabbits, and rats (data not shown). These lesions were marked by erythema surrounded by a ring of ischemia. The time course of HLT action on guinea pig skin is shown in Fig. 1. Small quantities of HLT (0.1 MID) produced transient ischemic lesions without erythema. Larger doses of HLT produced larger

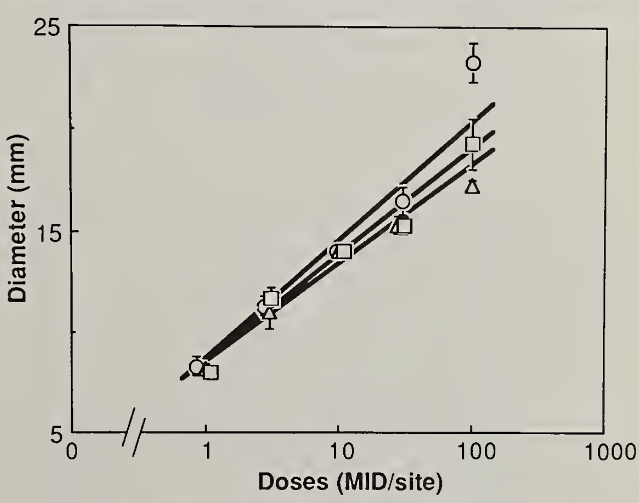

FIG. 2. Effect of endotoxin on ischemia-inducing activity of purified HLT. The indicated quantities of HLT in the absence (□) or presence of 30 (△) or 300 (○) ng of endotoxin in a total volume of 0.1 ml were injected into the shaved backs of guinea pigs. After 8 h, the diameter of the ischemia produced was measured. Each symbol with a vertical line represents the mean ± standard deviation of triplicate determinations. The lines determined by each set of points were not significantly different ($P > 0.05$). Reproduced with permission of *Infection and Immunity* (15).

lesions which appeared as erythema surrounded by ischemia.

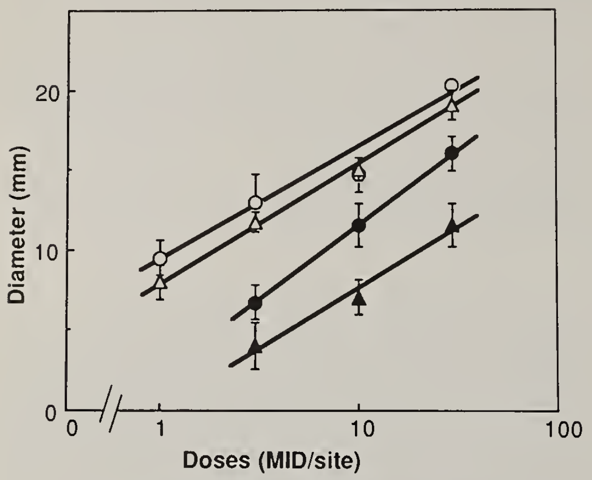

FIG. 3. Effect of BSA on ischemia- and erythema-inducing activities of purified HLT. HLT (1 to 30 MID/site) either with (○ , ●) or without (△ , ▲) BSA (1 mg) was injected in a total volume of 0.1 ml. The diameters of the ischemic (open symbols) and erythemic (closed symbols) lesions were measured at 8 and 24 h after the injection, respectively. Each symbol with a vertical line represents the mean ± standard deviation of triplicate determinations. The lines determined by the open symbols were not significantly different ($P > 0.05$). The lines determined by the closed symbols were significantly different ($P < 0.05$). Reproduced with permission of *Infection and Immunity* (15).

Kurokawa et al. (14) have previously described an effect of endotoxin on the size of hemorrhagic lesions produced by HLT preparations in guinea pigs. The present research therefore examined the ability of endotoxin to change the nature of the skin lesions produced by HLT. While endotoxin alone produced no lesion in guinea pig skin (data not shown), endotoxin in combination with HLT produced hemorrhagic lesions surrounded by a ring of ischemia (6). The time required for the appearance of hemorrhagic lesions induced by HLT in combination with endotoxin was shorter than that required for the appearance of erythemic lesions induced by HLT in the absence of endotoxin. This action is likely due to the endotoxin's lipid A portion since synthesized lipid A in combination with HLT produced hemorrhagic skin lesions (6). Although, the ability of HLT to induce hemorrhagic necrosis depended on the presence of endotoxin, the size of the ischemic lesion produced by HLT was not affected by this molecule (Fig. 2). Also, the time required for the appearance of ischemic lesions was not affected by the presence of endotoxin (data not shown).

These data suggest that what was previously considered a hallmark of HLT action, the hemorrhagic necrosis of guinea pig skin, is in fact due to HLT in combination with endotoxin. HLT alone produces ischemic lesions in guinea pig skin, an effect which may be due to the toxin's vasoconstrictive properties (7, 8, 9).

Since crude preparations of HLT also contain contaminating protein, we examined the effect of exogenously added protein (BSA) on HLT's ability to induce skin lesions. As shown in Fig. 3, HLT produced a larger erythemic lesion in the presence of BSA, but the diameter of ischemia was the same as that observed in its absence.

These results suggest that exogenous agents affect the type of skin lesion produced by HLT. Therefore, pure preparations of HLT cannot be reliably assayed in guinea pigs by a method in which hemorrhagic necrosis is measured. Instead, the diameter of the ischemic lesion produced by HLT could be used as a measure of the toxin's activity. Although the diameter of the ischemic lesion produced by HLT is dependent on the volume of sample injected (data not shown), the diameter of the ischemic lesion is independent of the presence of other agents and is linear with HLT dose. If the injection volumes of HLT preparations are standardized, the size of the ischemic lesions produced by the toxin can be used as a reliable measure.

Homogenize Tissue
⇓
Centrifuge (27,000 Xg) and Collect
Low Density Fraction (LDF)
⇓
Extract LDF with Methanol
⇓
Separate Extract by Reverse Phase
Chromatography
⇓
Obtain Two Fractions (F1+F2)
with Inhibitory Activity
⇓
Isolate Purified F1 and F2 after Repeating
Reverse Phase Chromatography

FIG. 4. Scheme for purification of substances which inhibit HLT.

Effect of lipids on HLT action. Whereas purified preparations of HLT produce ischemic lesions in guinea

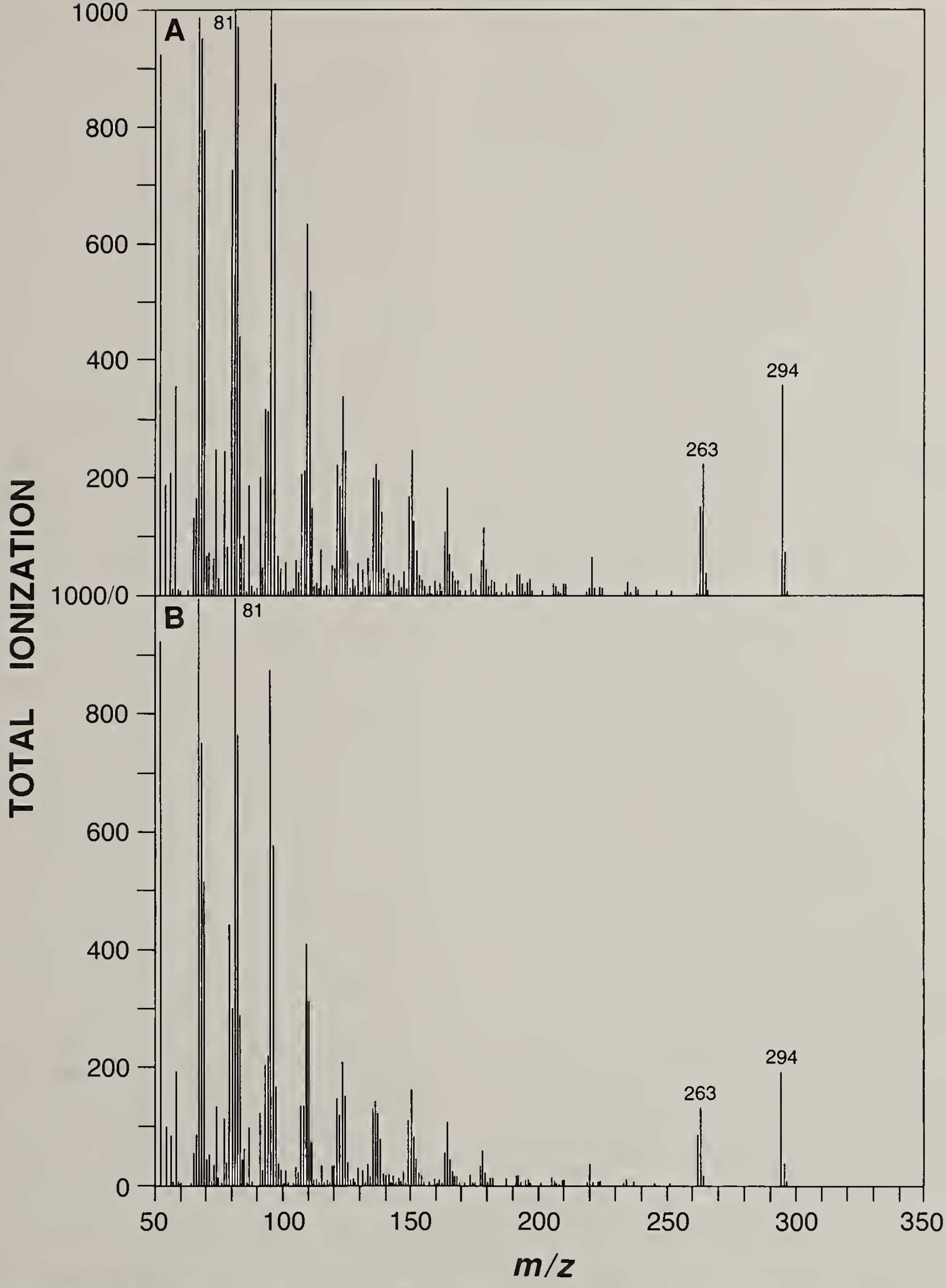

FIG. 5. Mass spectrometry pattern of F1 methyl ester. F1 methyl ester (A) or linoleic acid methyl ester (B) was analyzed by mass spectrometry as described in Materials and Methods.

pig skin, the same preparations produce hemorrhagic lesions in suckling mice and have no effect on the skin of adult mice. The molecular basis for these differences in HLT activity is not understood. We therefore investigated the possibility that the skin of adult mice might contain a substance which inhibits HLT and thus makes the animal resistant to its lesion-inducing activity.

Homogenates of adult mouse skin layer were obtained and were found to inhibit the ability of HLT to induce lesions in guinea pig skin (Fig. 5). In contrast, homogenates of liver, heart, lung, kidney, or skeletal muscle from adult mice had no affect on HLT activity. The inhibitory factors found in skin homogenates were purified as outlined in Fig. 4. After adding 1 ml of PBS per g wet weight of tissue, the mixture was homogenized in a glass tissue grinder. The homogenate was centrifuged at 27,000 x g for 20 min at 20°C, and the preparation separated into four layers: a low-density layer, a semi-solid layer, an aqueous layer, and a precipitate. The low-density fraction which contained HLT-inhibitory activity was collected by a capillary pipet. This fraction was extracted five times each with 2 ml of methanol per 1 gram of the preparation. Methanol extracts were pooled and concentrated by evaporation under nitrogen stream to obtain a final volume of 1 ml/g of low-density fraction. The concentrated methanol extract which contained the inhibitory activity was chromatographed on a high-pressure liquid chromatography reverse phase column (TSK gel ODS-80T_M, 4.6 mm by 250 mm, C_{18} end-capped column, Tosoh Co., Tokyo, Japan) with a pre-column (Toyopak ODS M, Tosoh Co.) using methanol as an eluent. Two fractions (F1 and F2) contained substances which inhibited the ability of HLT to induce ischemic lesions in guinea pig skin. The recovery of the inhibitory activity in these fractions was 68%. One µg of dry weight of F1 completely inhibited HLT activity at 300 MID. The specific activity of F1 was three times higher than that of F2.

F1 and F2 were also isolated from the skin layers of 2- to 3-week-old mice, rats (400 to 450 g), and guinea pigs (500 to 600 g) but not from suckling mice, smaller rats (150 to 200 g), or smaller guinea pigs (300 to 350 g). Both F1 and F2 inhibited the ability of HLT to induce skin lesions in different animal species; however, these factors did not alter the type of skin lesion produced.

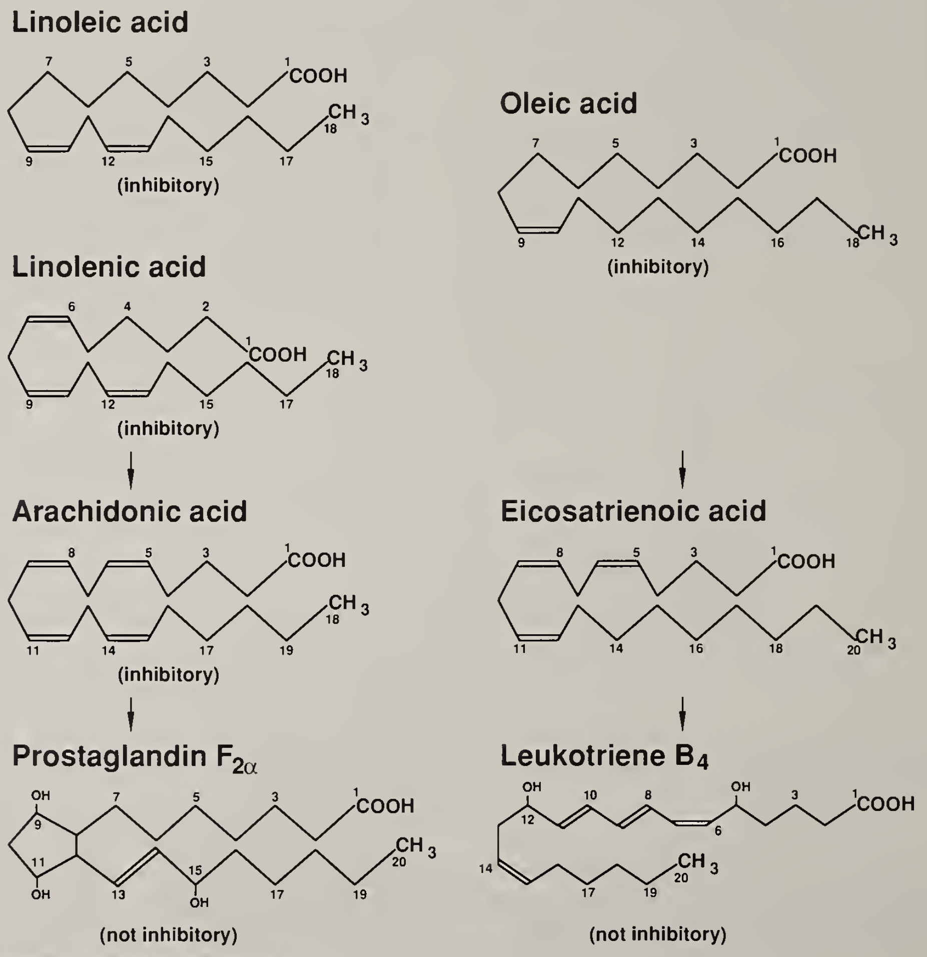

FIG. 6. Structures of unsaturated free fatty acids.

When F1 was analyzed by electron impact mass spectrometry using a high-sensitivity mode, its molecular weight was determined to be 280.239, a molecular weight very similar to that of linoleic acid (280.240). Moreover, the chemical formula estimated by a statistical analysis was $C_{18}H_{32}O_2$. Mass spectrophotometric analysis of the methyl ester of F1 revealed a fragmentation pattern identical to that of the methyl ester of linoleic acid (Fig. 5). F1 was therefore identified as linoleic acid.

F2 was analyzed next by mass spectrometry using a sensitive mode. Its fragmentation pattern indicated the presence of many methylene structures in the molecule. When the methyl ester of F2 was subjected to gas chromatography, two peaks (F2a and F2b) were obtained, the areas of which had a ratio of 4:6. These species were identified as palmitic and oleic acid methyl esters, respectively, using mass spectrometry.

Since these results suggested that the inhibitory factors found in skin layer were free fatty acids, we examined pure commercial preparations of fatty acids for their ability to inhibit HLT action. Pure preparations of oleic and linoleic acids were shown to have inhibitory effects on HLT activity; palmitic acid, however, did not have an inhibitory effect. Several other unsaturated fatty acids, including linolenic and arachidonic acids, inhibited the ability of HLT to induce ischemic lesions in guinea pig skin. The structures of some of these unsaturated fatty acids are shown in Fig. 6. Saturated fatty acids (except for high doses of lauric and myristic acids) had no effect on HLT activity. Fatty alcohol, neutral lipids, phospholipids, and cholesterol also had no effect on HLT activity. Prostaglandin $F_{2\alpha}$, which is synthesized from linoleic or linolenic acids via arachidonic acid (Fig. 6), and leukotriene B_4, which is synthesized from oleic acid via eicosatrienoic acid, do not inhibit HLT action.

The mechanism by which fatty acids inhibit HLT remains unknown. Possibly, fatty acids and HLT might affect the same biochemical pathway in eukaryotic cells although in opposite manners, resulting in inhibition of HLT activity. Alternatively, fatty acids might bind to a specific site on the HLT molecule, thus blocking a critical function of the toxin. Fatty acids also might simply act as amphiphiles which bind to the toxin and alter its conformation. While further studies needed to determine the molecular mechanism by which fatty acids inhibit HLT, the data reported here suggest that at least some of the differences in susceptibility of animals to the skin-lesion-inducing activity of HLT may be due to differences in the concentration of fatty acids in the skin layer.

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DISCUSSION

DR. GOLDMAN: When you had looked at this earlier with tracheal smooth muscle, you did not see any activity of the heat labile toxin and I think your speculation is now that there are cells that prevented access of the toxin to the smooth muscle tissue. Is it possible that the tracheal mucosa actually has a lot of these fatty acids which may be inhibitory to the action of the toxin and that has to be cleared away first as well?

DR. ENDOH: I do not think the tissue in the trachea has enough free fatty acid to inhibit HLT activity but we had not done yet to measure the fatty acids in the tissues. That is a good point.

DR. GUPTA: In the abstract of your paper, you mentioned that lipid A has an effect on HLT to produce hemorrhagic lesions. I want to know that if lipid A only from the *Bordetella pertussis* LPS will do this or if any lipid A can have the effect.

DR. ENDOH: Any lipid A can help the HLT action to cause hemorrhagic lesions.

DR. GUPTA: What portion of the lipid A, hydrophobic or hydrophilic, affects that?

DR. ENDOH: I do not think so. Our lipid A has activity to cause increase of permeability in the skin. That might help HLT activity to cause the hemorrhagic lesion.

DR. GUPTA: Are you sure that the fatty acids inhibit the effect of the HLT and the lipid A? On lipid A there are fatty acids in the hydrophobic region. If they bind to the HLT, they do not inhibit the effect of HLT.

DR. ENDOH: We do not know yet how the hydrophobicity of lipid A can act on HLT.

DR. HEWLETT: Once you showed the inhibitory effect of the fatty acids inducing skin lesions by HLT, you suggested that, the fatty acids might be acting by an effect on the target cell or an effect on the toxin. Did you go back and look at whether fatty acids are inhibitory of other effects of HLT like contraction of smooth muscle cells?

DR. ENDOH: That is a good point. If you add free fatty acids which can inhibit HLT activity in vivo to an HLT solution and then add the mixture to smooth muscle cells in culture, you will not see any contraction. The free fatty acids can inhibit the contraction.

DR. THOMAS: There appears to be no evidence that bronchial constriction is an important component of pertussis and albuterol does not seem to make any difference to the clinical condition of pertussis. How do you fit those facts into your thesis that HLT is an important cause of smooth muscle constriction in pertussis?

DR. ENDOH: With the pertussis infected bronchus, many kinds of tissues cover the tracheal smooth muscle cells or vascular smooth muscle cells. Therefore HLT cannot get into either the vascular or tracheal cells' smooth muscle cells.

MR. ZHANG: How much protein is equivalent to one MID?

DR. ENDOH: Around 0.3 nanogram protein.

DR. BEMIS: It has been suggested using some *Bordetella bronchiseptica* mutants that dermonecrotic toxin may be a common denominator in enhancing colonization by *Pasteurella multocida*. Have you studied the binding activities of your purified toxin to different species of cells, including perhaps other bacteria?

DR. ENDOH: We examined purified HLT from *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. The molecular weights are identical.

Construction of Genetically Detoxified Pertussis Toxin

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The conventional vaccine against whooping cough, composed of whole *Bordetella pertussis* bacterial cells, is highly efficacious in preventing disease. However, it is not widely used because immunization with this vaccine is associated with an excessive frequency of local and systemic side effects. Consequently, there are over 60 million cases of pertussis and more than half a million deaths from pertussis each year. Many *B. pertussis* antigens have been purified and tested for safety and efficacy in animal models and human clinical trials in an effort to develop safer and less reactogenic vaccines. The results have shown that vaccines containing chemically detoxified pertussis toxin (PT), either alone or combined with other antigens, are less reactogenic and have an efficacy similar to that of the whole-cell vaccine in preventing severe disease in children (3). In spite of the efficacy shown in clinical trials, acellular vaccines containing PT have not yet been licensed because doubts remain as to their safety and mechanism of protection.

During a large-scale clinical trial carried out in Sweden, chemically detoxified PT showed reversion to toxicity. To solve this problem, the study here reported developed an alternative method for detoxifying PT. We have modified two codons in the PT gene to obtain a *B. pertussis* strain which secretes a PT molecule (PT-9K/129G) naturally devoid of toxicity.

PT, a protein composed of five different subunits, can be divided into functional domains A and B. A (composed of the subunit S1) is an enzyme that modifies some G proteins of eukaryotic cells by ADP ribosylation, altering their ability to respond to exogenous stimuli. B (composed of subunits S2, S3, S4, and S5 in 1:1:2:1 ratio) is a nontoxic oligomer that binds to receptors on the surfaces of eukaryotic cells and delivers the S1 subunit across the plasma membrane. The genes coding for the five subunits of PT are clustered in an operon (6). After unsuccessful trials to express the PT operon in *Escherichia coli*, the individual subunits were expressed as either natural or fusion proteins in *E. coli* and *Bacillus subtilis* and used to immunize mice and rabbits. None of the subunits either alone or in any combination (i) induced toxin-neutralizing antibodies or (ii) protected mice from intracerebral challenge with virulent *B. pertussis* (5). This result was unexpected because both the natural toxin and the anti-S1 monoclonal antibodies had been shown to be effective in both assays. Further studies showed that the recombinant subunits had a conformation different from the natural molecule and were therefore able to induce only nonprotective antibodies.

Genetic detoxification of PT. The failure to induce protective immunity with recombinant subunits suggested that the ideal vaccine should be a PT molecule whose toxicity had been eliminated by genetic manipulation of the gene coding for subunit S1. To do so, several recombinant S1 molecules containing amino acid substitutions were generated and tested for their enzymatic activity. Substituting either Arginine-9, Aspartic Acid-11, Arginine-13, Tryptophan-26, or Glutamic Acid-129 was found to reduce the enzymatic activity to undetectable levels (1, 2, 7). Each of the above mutations was then introduced into the chromosome of *B. pertussis* strains whose wild-type genes had been deleted. These new *B. pertussis* strains were found to produce molecules indistinguishable from PT when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecules had a toxicity that ranged from 0.1% to 100% of wild-type PT. Since even 0.1% toxicity is much too high for a molecule that is to be used in a vaccine, we combined some of the above mutations to obtain PT double mutants that were at least 10^6 times less toxic than wild-type PT (8). Such nontoxic molecules were ideal candidates for new vaccines provided that they had main-

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* Presenting author.

TABLE 1. In vivo and in vitro properties of PT-9K/129G compared with purified native PT

Property	PT-9K/129G	PT
CHO cell clustered growth (ng/ml)	> 5,000	0.005
ADP ribosylation (µg)	> 20	0.001
Mitogenicity (µg/ml)	0.1 - 0.3	0.1 - 0.3
Hemagglutination (µg/well)	0.1	0.1
Affinity constant (anti-S1) [Ka(L/mol)]	6.1 x 10^8	2.4 x 10^8
Affinity constant (anti-PT) [Ka(L/mol)]	9.8 x 10^9	2.0 x 10^{10}
Histamine-sensitization (µg/mouse)	> 50	0.1 - 0.5
Leukocytosis stimulation (µg/mouse)	> 50	0.02
Anaphylaxis potentiation (µg/mouse)	> 7.5	0.04
Enhanced insulin secretion (µg/mouse)	> 25	< 1
In vivo acute toxicity (µg/kg)	> 1,500	n.d.

n.d., not determined.

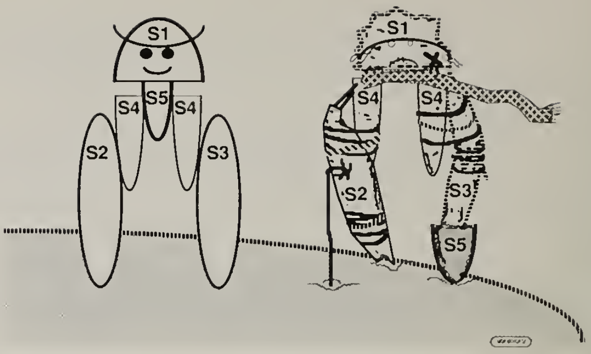

FIG. 1. Schematic representation of the genetically inactivated PT-9K/129G (left) and of the chemically inactivated PT (right). PT-9K/129G is nontoxic and maintains all the protective epitopes of the native toxin. Consequently, very low doses are enough for efficient immunization. In marked contrast, chemical treatment heavily modifies toxin conformation (right). The chemically modified molecule (which theoretically can still revert to toxicity) induces mainly nonprotective antibodies; therefore, high doses are required for efficient immunization.

tained the correct B- and T-cell epitopes and were able to induce protective immunity in animal models. The nontoxic double mutant shown in Table 1 was found to (i) have the same B- and T-cell epitopes as wild-type toxin, (ii) induce toxin-neutralizing antibodies, and (iii) protect mice from intracerebral challenge (4).

Immunogenicity of genetically and chemically detoxified PT molecules. Formaldehyde and glutaraldehyde are usually used to detoxify toxins for vaccine purposes. To find out whether PT-9K/129G is more immunogenic than chemically inactivated PT molecules, PT-9K/129G was treated with 0.07% and 0.42% formaldehyde for 48 hours at 37°C and the in vitro properties and immunogenicity of the resulting molecules compared.

As shown in Table 2, formaldehyde treatment abolished the hemagglutinating property of PT-9K/129G, decreased its affinity for anti-PT gamma globulins, and masked the epitope recognized by the protective monoclonal antibody 1B7. The immunogenicity of PT-9K/129G did not decrease after formaldehyde treatment. The enzyme-linked immunosorbent assay (ELISA) titers of the sera obtained from guinea pigs immunized with natural or formalin-treated molecules were almost identical (Table 2). In marked contrast, the ability of the sera to neutralize PT in the Chinese hamster ovary (CHO) assay was dramatically lower when chemically treated

TABLE 2. Effect of formaldehyde treatment on properties of genetically inactivated PT mutant PT-9K/129G

Formaldehyde	Hemagglutination	Affinity Constant		Immunogenicity		Vaccine Potency
(%)	(µg/well)	Polyclonal gamma globulins	Monoclonal 1B7	(Guinea pigs immunized with 3 µg of antigen)		Mice survival after immunization with 5 µg of antigen
				ELISA titer (Absorbance)	CHO titer	Intracerebral challenge
0.00	0.5	1.15 x 10^9	5.5 107	3.5	2560	13/16
0.07	4.0	5.26 x 10^8		3.1	160	8/16
0.42	>10.0	6.75 x 10^7		3.3	80	1/16

TABLE 3. Serum antibody responses in adult volunteers receiving PT-9K/129G vaccine or placebo

	Time (Day)	Experimental Groups	
		Vaccine	Placebo
		(Geometric Mean)	
Neutralizing titer (CHO assay units)	1	12.8	13.6
	+30	12.8	1,810.2
	+72	11.7	1,974.0
Antibody titer anti-PT (ELISA units)	1	6.5	6.7
	+30	8.1	496.5
	+72	7.9	522.4

molecules were used for immunization. In the intracerebral challenge assay, the formalin-treated molecules were remarkably less potent than natural PT-9K/129G (Table 2).

These results show that chemical treatment of toxins for vaccine production induces profound changes in the antigenic properties of the molecules. These changes do not alter the total amount of antibodies induced; however, they dramatically alter the quality of the antibodies obtained. As a result, large quantities of chemically treated molecules are required to induce a protective response. Under these conditions, the immune system mainly produces antibodies against nonprotective epitopes (or antibodies with a lower affinity for protective epitopes), and the cellular immunity is overstimulated. Both conditions may favor the appearance of untoward reactions of the Arthus- or delayed-type hypersensitivity.

In conclusion, PT-9K/129G has the same conformation as native proteins (Fig. 1) and is much more efficient than chemically treated toxins in inducing protective immunity. These properties demonstrate that molecular genetics can provide new and more efficient tools to inactivate toxins for vaccine use.

Clinical studies. After extensive studies in animal models which have shown that PT-9K/129G is nontoxic, immunogenic, and able to protect mice from infection with virulent *B. pertussis,* the researchers tested PT-9K/129G in adult human volunteers. The results of this study are summarized in Table 3 (9) and described in detail elsewhere in this volume. The PT-9K/129G vaccine was safe and induced a great increase in antibody titers against PT both in ELISA and CHO neutralization assays (Table 3.) The titers of PT-specific antibodies were higher than those reported in similar studies using higher doses of chemically detoxified PT. In particular, the ratio between toxin-neutralizing titers and total anti-PT ELISA titers was the highest so far reported, suggesting that in humans too, immunization with a molecule that has not been chemically modified induces antibodies with a higher affinity for native PT.

Given the successful phase-I study in adult volunteers, the vaccine is now being tested in 3- and 15-month-old children. The results to date confirm the excellent properties of the new pertussis vaccine.

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DISCUSSION

DR. BURNS: You showed that in some of your mutants that did not make the S-1 submit or did not properly make it, that the B oligomer could get out of the cell. How efficient is that process?

DR. RAPPUOLI: That process is very inefficient. In the supernatant, we find at least 10 times less B oligomer than wild-type toxin so we assume that the S-1 subunit is necessary for efficient export of the entire toxin. In case you do not have S-1 subunit, you still get the B-oligomer in the supernatant but that is much less than you will have so the process is very inefficient.

DR. BURNETT: Did you have an opportunity to look at the reversion rate of your single and double mutants in *Bordetella*?

DR. RAPPUOLI: We never looked at the reversion rates of the single mutants. For the double mutants, basically we grow them in fermentors up to 300 liters and after each fermentation we measure or we tried to measure whether we see any reversion and so far we have not settled that. In theory, since we have used all of the bases to change two amino acids, the theoretical reversion should be in the range of 10^{-28} so in theory it should be difficult to get to reversion.

DR. SCOTT: In one of your slides comparing the acellular with the whole cell vaccine, I was a little confused about the doses and the results. I may not be reading this correctly, but with decreasing doses injected, you have fewer survivors?

DR. RAPPUOLI: Yes, those depend on survivors.

DR. SCOTT: The less injected, the fewer survive? It should be just the opposite, is that correct?

DR. RAPPUOLI: No. If you inject with 30 micrograms, all survive. If you inject with 25 micrograms, only few mice will survive. It is just dose dependent.

DR. SCOTT: Okay, thank you.

DR. PARKER: Would you comment on the advisability or desirability of retaining the mitogenicity in the toxin presumably due to the B subunit? Is that good, bad or do we not know yet?

DR. RAPPUOLI: I did not ask the question of whether it is good. I just asked the question whether it is bad and the answer is it is not bad. First in animals and then in humans, we basically studied any of the parameters which are known to be affected by the mitogenicity and we did not observe any change so we think it is very safe. We published that in the Journal of Preventive Medicine this month.

DR. GUPTA: Did you calculate the potency by the intracerebral challenge method in international units in a human dose?

DR. RAPPUOLI: The question is whether we know the potency of our vaccine?

DR. GUPTA: Yes.

DR. RAPPUOLI: Obviously you cannot compare number of cells against micrograms of protein, so you have to compare the human dose versus human dose. If you compare the toxin alone, our mutant toxin alone can be five to ten times less potent than the whole cell vaccine. However, if you add 69 kDa and FHA you can get vaccines which are even more potent than the cellular vaccine.

Dr. STAINER: Is there any difference in the growth requirements and the relative growth of your mutants and the normal strains?

DR. RAPPUOLI: Absolutely not. Their physiology seems to be identical to the wild type strain.

DR. Y. SATO: If you take your mutant toxoid and our purified natural toxoid and adsorbed both on aluminum hydroxide and immunized mice at the same time, there was no difference in potency using the intracerebral challenge method. Potency as compared by microgram protein for each vaccine. There is no difference in potency for pure native toxoid and pure mutant toxoid. But you mention that the mutant toxoid is more potent. What does that mean?

DR. RAPPUOLI: What is meant is that the vaccines which have been made by chemical detoxification by other manufacturers have not been available to me, so I could not compare them. Instead, I have treated the toxin or the mutant toxin with formaldehyde and checked the immunogenicity and we find that when you treat with formaldehyde you get a great reduction of immunogenicity. But you might be right. I have never been able to test your molecules or those made by other labs so I cannot comment on that.

Molecular Biological Studies on the Structure-Function Relationship of Pertussis Toxin and Filamentous Hemagglutinin

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Virulent *Bordetella pertussis* is characterized by the coordinated production of several virulence factors. These factors are responsible for the different steps needed to establish disease in the human host and include those that mediate attachment to the ciliated cells in the respiratory tract and toxins that cause physiological changes in the infected host (61). Among them, pertussis toxin (PT) and filamentous hemagglutinin (FHA) are of great importance in the pathogenesis of whooping cough. *B. pertussis* mutants affected in the production of PT are significantly less virulent in various animal models (62). Several in vivo effects of virulent *B. pertussis* can be reproduced by administering purified PT to mice (44). The virulence of other bacterial pathogens can be enhanced by the addition of purified FHA (58). Furthermore, PT and FHA induce protective immunity against challenge in mice (33, 52) and have been shown to be good protective antigens for preventing whooping cough in children (1, 51). Although other factors are clearly required for virulence, and immune reactions against them probably would prevent disease, PT and FHA are to date the only antigens that have been shown to provide protection in humans as well as in animal models.

PT is a hexameric protein composed of five dissimilar subunits structured in an A-B model (57). The subunits, named S1 to S5 according to their decreasing molecular weights, are arranged in two dimers: dimer 1, composed of S2 and S4, and dimer 2, composed of S3 and S4. These two are connected by subunit S5, thereby constituting the B oligomer; the A protomer is represented by the S1 subunit. Via the B oligomer the toxin binds to the receptors on the target cells. With the help of the B oligomer, the A protomer is introduced into the cytosol of the target cells. In the cytosol, S1 expresses enzymatic ADP-ribosyltransferase activity on signal-transducing GTP-binding proteins. The structural gene of PT has been cloned (36, 46) and sequenced (39, 46). Subunits S2 and S3 were found to be 70% identical in their amino acid sequence. The S1 subunit shares two regions of sequence similarity with the ADP-ribosyltransferase moiety of cholera toxin (CT). At least one of these regions appears to be important for enzymatic activity (7, 14); it is also part of an important protective epitope (14).

FHA is a very large protein that assembles into filamentous rods when purified from *B. pertussis* culture supernatants (64). Purified FHA appears heterogeneous when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25). Its antigenic structure is less well studied than that of PT: its complete structural gene has only recently been cloned and sequenced (17, 20). The FHA sequence revealed the presence of two RGD sites, one of which is involved in the binding of FHA to integrin-like molecules of target cells (49, 50). Although evidence exists for another, carbohydrate-binding site on FHA (59), this site has not yet been identified.

The present study analyzes the structure-function relationship of both PT and FHA. Understanding this relationship may help provide elements for a rational design of future safe and efficacious pertussis vaccines.

MATERIALS AND METHODS

Sequence analyses. The analysis of nucleotide and amino acid sequences was performed using the University of Wisconsin software package (19). Homology searches were done with NBRF (release 21) and GenBank (release 60). Hydrophobicity plots were generated using the method described by Kyte and Doolittle (34).

Purification of rS1d. The researchers purified rS1d and its analogs from cell lysates of *Escherichia coli* TG1 (Amersham, United Kingdom) harboring recombinant plasmids containing the different rS1d genes under the control of the *lac* promoter. After induction, cell lysis, and centrifugation as previously described (38), the supernatant fraction was first applied onto a DEAE-cellulose column, as described elsewhere (40). The fractions containing rS1d-related material were pooled and their salt concentration was adjusted to 500 mM NaCl. The fractions were then applied onto a phenyl-sepharose column, previously equilibrated in 500 mM NaCl, 25 mM Tris.Cl (pH 7.5). The column was then extensively washed in the same buffer, and the rS1d-related material eluted in 10 mM Tris.Cl (pH 9.5). The fractions containing rS1d-related proteins were pooled, dialyzed against 10 mM Tris.Cl (pH 7.5), and lyophilized until further use.

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Enzyme activities. ADP-ribosyltransferase activities were carried out as described by Cieplak et al. (14) or Locht et al. (38). NAD glycohydrolase activities were performed as described by Locht et al. (38).

Recombinant DNA techniques. Site-directed mutagenesis was carried out using the oligonucleotide-directed in vitro mutagenesis system (Amersham) under the conditions recommended by the supplier. Oligonucleotides were synthesized on an automated solid-phase synthesizer. DNA sequencing was carried out with the Sequenase DNA Sequencing kit (United States Biochemical Corporation, Cleveland, Ohio), also as per manufacturer specifications. All standard recombinant DNA techniques used were those described by Maniatis et al. (40). Bacterial conjugation was performed as described by Antoine and Locht (3).

Other analytical procedures. SDS-PAGE with 12.5% slab gels was performed as described by Laemmli (35). Western blot analysis using anti-S1 or anti-S2 monoclonal antibodies was performed as described earlier (38). Western blots using anti-FHA antibodies kindly provided by I. Heron (Statens Seruminstitut) and E. Simoens (SmithKline Biologicals) were carried out in a similar fashion. The enzyme-linked immunosorbent assays using monoclonal antibodies described by Francotte et al. (23) were detailed earlier (3). Cytotoxicity of PT and PT cross-reactive mutants (PT-CRM) on Chinese hamster ovary cells was analyzed as described by Hewlett et al. (24) with minor modifications as described by Antoine and Locht (3). Bacterial strains, growth conditions, and DNA extraction were described earlier (3).

RESULTS AND DISCUSSION

The S1 subunit of PT catalyzes ADP ribosylation of signal-transducing G proteins, using NAD as the ADP-ribose donor substrate (32). In the absence of G proteins, S1 catalyzes the transfer of ADP ribose to a water molecule; this is the NAD glycohydrolase reaction (31). From a mechanistic point of view, the enzyme contains at least three active sites: the NAD-binding site, the G protein-binding site, and the catalytic site. In the actual protein structure the three sites may overlap to a certain extent.

Role of the carboxy-terminal region of S1 in ADP-ribosyltransferase activity. Several groups have produced active S1 subunit in *E. coli* and have found it to accumulate as insoluble particles (5, 8, 38, 45). The hydrophobicity plot (Fig. 1) shows that the subunit's major hydrophobic regions are located in the carboxy-terminal part of the molecule. By analogy to CT, it was speculated that this carboxy-terminal region might be involved in binding the S1 subunit to the B oligomer rather than in enzymatic activity (9). Preliminary results indicated that deletion of the 47 carboxy-terminal amino acids did not abolish enzymatic activities (38). However, this deletion rendered the recombinant S1 molecule (rS1d) soluble in low-salt aqueous buffers (3). The solubility made it possible to purify rS1d to apparent homogeneity by two chromatographic steps (Fig. 2). Comparison of the purified rS1d with full-length recombinant S1 (rS1) showed that the NAD glycohydrolase activities of rS1 and rS1d were indistinguishable with respect to the specific activities and apparent K_m values for NAD. In contrast, the level of ADP ribosyltransferase of rS1d was significantly reduced as compared to that of

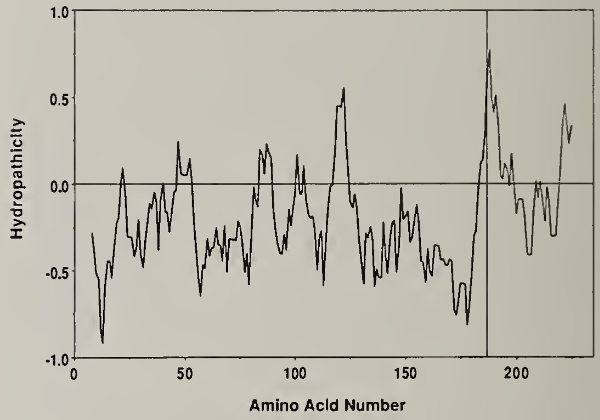

FIG. 1. Hydropathicity analysis of the PT S1 subunit. This analysis was performed using the method of Kyte and Doolittle (33). The vertical line indicates the point at which rS1d is truncated (amino acid residue 187). Positive values denote increasing hydrophobicity.

TABLE 1. Comparison of the enzymatic activities of rS1 and rS1d

S1 analog	ADP ribosyltransferase (%)	NAD glycohydrolase (%)	K_m (NAD) µM	CHAPS (%)
rS1	100	100	16	30
rS1d	5	100	11.6	35

CHAPS, 3-([3-Cholamidopropyl]-dimethylammonio)-1-propane-sulfonate.

rS1 (Table 1). This finding suggests that the carboxy-terminal region of the S1 subunit is not involved in NAD-binding and catalysis, but rather in the interaction of the enzyme with its donor-substrate G protein. Further comparison of rS1d with rS1 indicated that their requirement for dipolar ions such as CHAPS for NAD glycohydrolase activity is very similar (Table 1), indicating that the carboxy-terminal deletion of S1 does not abolish the L-site identified by Kaslow et al. (29).

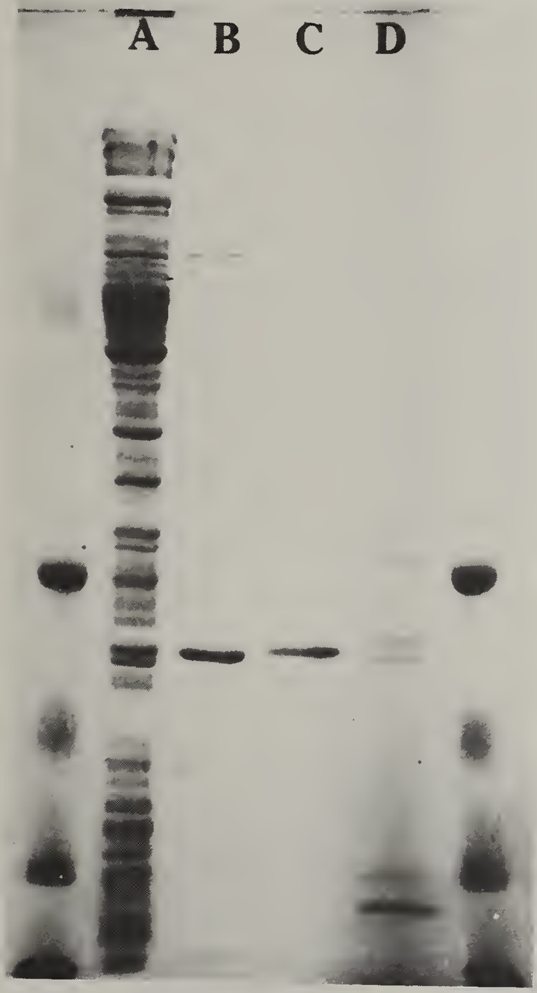

FIG. 2. Purification of rS1d. The recombinant protein rS1d was purified from *E. coli* cell lysates by two chromatographic steps as described in text. Different fractions were then analyzed by SDS-PAGE and Coomassie-blue staining. Lane A contains the total supernatant fraction after cell lysis. Lane B contains the pooled fraction after DEAE-cellulose chromatography. Lane C contains the pooled fraction after phenyl-sepharose chromatography, and lane D contains purified PT (List Biological Laboratories, Campbell, Calif.).

Role of Cysteine 41 in S1 NAD glycohydrolase activity. The enzyme activities of the holotoxin can be detected when the S1 subunit is dissociated from the B oligomer by the use of denaturing agents such as urea or reducing agents such as DTT in the presence of dipolar ion detergents and ATP (11). Isolated S1 subunit expresses its enzyme activities after activation by thiol (43), suggesting that the single disulfide bond of S1 needs to be reduced to expose at least one of the active sites. Chemical modification of the two Cysteines after reduction of the protein significantly reduced enzymatic activities (28). Furthermore, NAD appeared to protect one of the Cysteines from modification, indicating that this protected Cysteine is in the vicinity of the NAD-binding domain (28). Evidence has been accumulated to indicate that the protected Cysteine residue is Cys-41 (12, 30).

Since rS1d contains only one of the two original Cysteine residues (Cys-200 was deleted), its NAD glycohydrolase activity was assayed in both the presence and absence of DTT. As shown in Fig. 3, the presence of DTT significantly increased the NAD glycohydrolase activity of rS1d. Western blot analysis using anti-S1 monoclonal antibodies showed that the electrophoretic mobility of rS1d did not change when electrophoresis was carried out in reducing conditions versus nonreducing conditions. This finding indicated that (i) rS1d does not undergo dimerization through a disulfide bond involving Cys-41 and (ii) a rather small group — too small to cause a difference in electrophoretic mobility — was blocking the sulfhydryl group of Cys-41 in rS1d; this blockage was sufficient to cause reduction in NAD glycohydrolase activity.

The role of Cys-41 in NAD glycohydrolase activity was further investigated by site-directed mutagenesis.

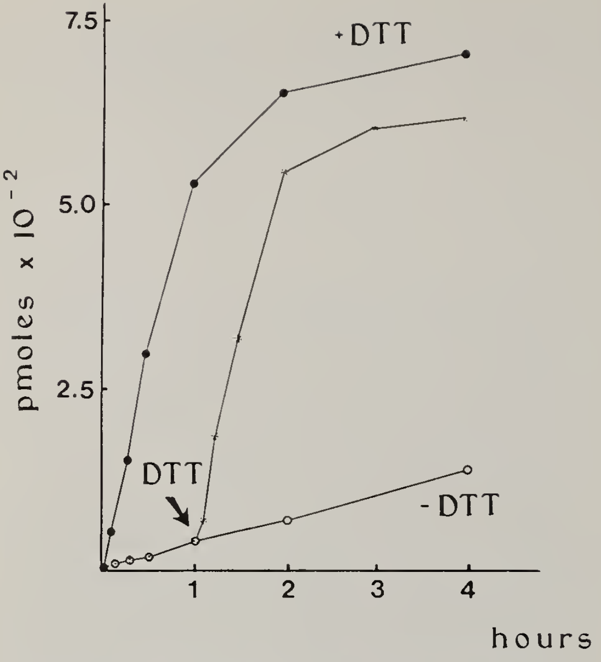

FIG. 3. Effect of DTT on rS1d NAD glycohydrolase activity. The NAD glycohydrolase activity of rS1d was assayed in the presence (●——●) or absence (○——○) of DTT. In a third sample (*——*), the NAD glycohydrolase activity was carried out in the absence of DTT for 1 h; DTT was then added as indicated by the arrow.

When Cys-41 was deleted, the enzyme activity was reduced to undetectable levels. However, when Cys-41 was replaced by 1Serine, Glycine, Proline or Asparagine, the activity was reduced to between 20 and 40% of residual activity. This finding indicated that the sulfhydryl group of Cys-41 is not essential for activity and that Cys-41 most likely is not a catalytic residue (40). Comparing the kinetic parameters of rS1d-C41G with those of rS1d showed that whereas the K_m value for NAD was increased in the mutant enzyme, the k_{cat} values were not significantly different (Table 2). Therefore, Cys-41 is not a catalytic residue but is located in close proximity to the NAD-binding site. The reduction of activity to residual levels of 1 to 2% when Cys-41 was replaced by Asp or Glu indicated that a negative charge at position 41 was more detrimental to activity than a different polar group. This detriment was possibly because of charge-charge repulsion with the donor substrate, suggesting that Cys-41 may be close to the phosphate subsite of the NAD-binding site (40).

Although these results did not help to identify a catalytic residue in S1, they did allow the NAD-binding site of the enzyme to be localized in the vicinity of Cys-41 between two regions that share sequence similarity with CT.

Identification of active-site residues in S1. Among the various ADP-ribosylating toxins, diphtheria toxin (DT) and Exotoxin A of *Pseudomonas aeruginosa* (ET) are the best studied with respect to their enzymatic activities. Glu-553 has been identified as a catalytic residue in ET by several criteria. First, photoaffinity labeling of ET with radioactive NAD resulted in the attachment of nicotinamide to Glu-553 (13). Second, the crystal structure of ET showed that Glu-553 is localized in the active-site pocket (2). Third, site-directed modification of Glu-553 greatly reduced enzymatic activity (21). Finally, some alterations at position 553 resulted in a significantly decreased k_{cat} value, whereas the K_m for NAD was not significantly altered (22). The mechanism of DT seems to be very similar to that of ET, and in both toxins an important Tryptophan residue is located five amino acids downstream of the catalytic Glu. Fluorescent quenching experiments have shown that this Tryptophan residue of DT is at the NAD-binding site (27).

Whereas rS1d contains several Glu residues, it contains only one Trp residue, Trp-26. To investigate whether the same residues are involved in the enzymatic activities of PT and ET despite the absence of statistically significant homology between the two toxins, Trp-26 of rS1d was altered by site-directed mutagenesis. Deletion of Trp-26 or its replacement by Threonine resulted in reduction of enzymatic activities to undetectable levels (37). Only its replacement by an aromatic amino acid such as Tyr allowed us to measure reliable enzyme activity. This finding indicated that the site chain of Trp-26 plays a more important role than that of Cys-41. Consequently, the kinetic parameters of rS1d-W26Y were compared to those of rS1d-C41G and rS1d. As shown in Table 2, the K_m for NAD was drastically increased in rS1d-W26Y as compared to rS1d-C41G and rS1d. Similar results were recently obtained by Cortina and Barbieri (16). On the other hand, the k_{cat} value for the NAD glycohydrolase activity was slightly increased when Trp-26 was replaced by Tyr, in contrast to the alteration of Cys-41. This finding indicated that Trp-26 is involved in NAD binding of S1 and is consistent with the idea that the amino-terminal portion of S1 encompassing the regions of sequence-similarity with CT is the NAD-binding domain (Fig. 4).

Whereas in DT and ET the critical Trp residue is in close proximity to the catalytic Glu residue, no Glu residue could be found close to Trp-26 in the S1 primary

TABLE 2. Comparison of kinetic parameters between rS1d, rS1d-C41G, and rS1d-W26Y

S1 analog	K_m (NAD) μM	k_{cat} min^{-1}	k_{cat}/K_m $min^{-1} \times \mu M^{-1}$	%
rS1d	11.6	0.50	43.1×10^{-3}	100
rS1d-C41G	29.4	0.42	14.3×10^{-3}	33
rS1d-W26Y	500	1.48	2.96×10^{-3}	6.87

structure. However, like DT and ET, PT can also be photoaffinity-labeled with radioactive NAD (4, 15, 16). Furthermore, like that of ET, S1 photolabeling is proportional to NAD binding (15). Analysis of radiolabeling after cyanogen bromide cleavage indicated that the incorporation of radiolabel occurs at a different location in the primary structure than the above-identified NAD-binding site. Two groups (4, 16) independently located the photolabeled Glu residue at amino acid position 129. Deletion of Glu-129 or its replacement by Asp using site-directed mutagenesis resulted in undetectable NAD glycohydrolase and ADP-ribosyltransferase activities (37). Together, these results suggest that (i) Glu-129 of S1 plays a similar catalytic role in enzyme activity as Glu-148 in DT and Glu-553 in ET, and (ii) despite the absence of significant homology between the different ADP-ribosylating toxins, the residues critical for enzymatic activity appear to be identical.

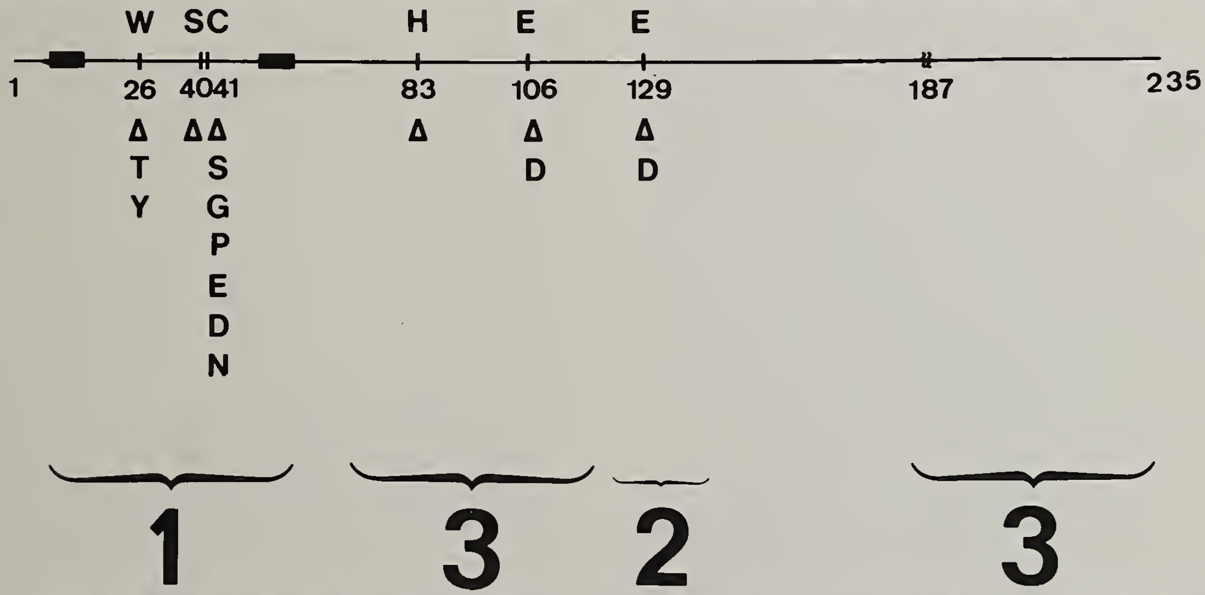

FIG. 4. Summary of the described mutations and structure-function model of S1. The S1 subunit is represented by the horizontal line. The ■■ indicate the two regions of S1 that share sequence similarities with CT. Mutations are indicated by the small vertical lines at their respective amino acid positions. The altered amino acids are indicated by their single letter codes. Amino acid substitutions studied are indicated by single letter codes below their respective positions in the primary structure. The ≈ line at position 187 indicates the point of truncation of rS1d. Proposed domains of the protein are indicated by [ ]. Domain 1 is the proposed NAD-binding site. Domain 2 contains the proposed catalytic residue. Domain 3 is proposed to be responsible for G protein-binding.

The important Trp and Glu residues in S1, unlike those in DT and ET, are located at distant sites in the molecule's primary structure. If Glu-129 of S1 is indeed a catalytic residue, it should be in close proximity to the NAD-binding site in the tertiary structure. To test this hypothesis, Glu-129 was replaced by Cysteine. Cys-129 was found to form a disulfide bond with Cys-41, previously shown to be located at the NAD-binding site. This finding indicated that there are at least no steric constraints to bring Glu-129 close to the NAD-binding site in the three-dimensional structure of S1.

Effect of the alteration of active-site residues on PT assembly. To study the effects of amino acid substitutions at the active sites of S1 on the assembly of the holotoxin as well as on cytotoxicity, a *B. pertussis* expression system was developed. Mutant PT genes were inserted into the low copy number plasmid pLAFRII and introduced by conjugation into *B. pertussis*. To ensure that only the PT gene on the incoming plasmid, and not the normally present chromosomal gene, would be expressed in the *B. pertussis* exconjugants, the wild-type gene was first deleted from the chromosome of *B. pertussis* Tohama I. The deletion was performed in two steps with the help of an integrating plasmid derived from pRTP1 (56) carrying the flanking regions of the PT gene. One of the resulting *B. pertussis* strains, BPRA, then was further analyzed. Southern as well as Western blot analyses showed that the PT gene was indeed deleted and that this deletion resulted in the absence of detectable production of PT (3).

The wild-type toxin gene was then inserted into pLAFRII and introduced into BPRA. Enzyme-linked immunosorbent assays indicated that the toxin gene was successfully expressed and its product assembled and secreted at levels comparable to those produced by the original *B. pertussis* Tohama I strain (3). Subsequently, the wild-type PT gene on pLAFRII was replaced by several mutant genes. First, the disulfide bond in the S1 subunit was abolished through mutations at codon 41. Cys-41 was either deleted or replaced by Glycine or Serine in these mutant proteins. In all three cases, S1-free B oligomer was produced and secreted by BPRA. These results indicated that S1-free B oligomer can be secreted by *B. pertussis* and that the disulfide bond in the S1 subunit is essential for the binding of this subunit to the B oligomer and its subsequent secretion.

No cell-associated S1 subunit was detected, suggesting that either the mutation at codon 41 prevents expression of this cistron or that, due to the absence of the disulfide bond in the subunit, it cannot assemble with the B oligomer and is therefore rapidly degraded. The second hypothesis seems more likely since in heterologous prokaryotic expression systems, there is no evidence for a deleterious effect of these mutations on expression of the S1 subunit gene (40). Furthermore, transcription of the gene in *B. pertussis* is not affected by the mutations, since the cistrons downstream of the S1 cistrons were efficiently expressed.

Similar results were obtained when the region coding for the carboxy-terminal part of the S1 subunit was deleted, indicating that both the carboxy-terminal part of the S1 subunit as well as its disulfide bond are required for the binding of S1 to the B oligomer (3).

A variety of single and double mutations resulting in changes of active-site residues were then introduced into BPRA to investigate their effect on assembly and secretion as well as on cytotoxicity. None of the PT-CRM analyzed showed any cytotoxic effect on Chinese hamster ovary cells, confirming that cytotoxicity depends on the ADP-ribosyltransferase activity of S1 (10).

The assembly and secretion of the PT-CRM were analyzed by enzyme-linked immunosorbent assays using haptoglobin-coated microtiter plates and subunit-specific monoclonal antibodies to quantify the presence of individual subunits on the haptoglobin-bound PT-CRM. Quantification using anti-S2, anti-S3, or anti-S4 monoclonal antibodies indicated stoechiometric amounts of these three subunits in the PT-CRM, indicating that B oligomers were stably assembled.

Unlike those of the B oligomer subunits, the amounts of detected S1 subunits varied in the different PT-CRM and were significantly and consistently less than in wild-type PT. However, the amounts of S1 subunits were higher than in PT-CRM in which the Cys-41 residue or carboxy-terminal region had been altered. These results suggest that S1 subunits in which Trp-26 or Glu-129 in the active site had been changed are still able to assemble to the B oligomer and to be secreted into the culture medium, but that the interaction of these S1 subunits with the B oligomer is less stable than that of wild-type S1. S1 subunits with a double mutation at Trp-26 and Glu-129 are even less stable than S1 subunits containing only single mutations. However, there are mutations at either codon that do not seem to affect assembly (47); this is consistent with the model wherein the active site of the S1 subunit is not exposed in the native toxin, but is shielded by the B oligomer. Indeed, expression of PT enzymatic activities requires prior dissociation of the S1 subunit from the B oligomer, which can be achieved by incubation of the toxin with DTT, ATP, detergents, and urea. Therefore, it appears likely that the active site is located at the side of the S1 subunit that interacts with the B oligomer.

Structure-function relationship of FHA. Much less is known about the structure-function relationship of FHA than of PT. The amino acid sequence of this important virulence factor has only recently been deduced from the nucleotide sequence of its structural gene (17, 20, 49). To elucidate the structure-function relationship of FHA, the cloning of its entire structural gene was undertaken. The *B. pertussis* genomic bank was probed with three different DNA probes. One probe contained the sequence adjacent to a Tn*5* insertion which resulted in an FHA⁻ phenotype of *B. pertussis* (63). The second probe was a synthetic oligonucleotide corresponding to one of the sequences determined after the cloning of the partial gene in a lambda expression vector (42). Finally, the third probe was an oligonucleotide derived from the back translation of the amino-terminal amino acid sequence of an FHA-related protein (26). Together these three probes covered a DNA region of over 20 kilobases that was cloned in four overlapping fragments (17).

The nucleotide sequence of the 3,514 5′ residues of the gene was determined. This sequence showed several interesting features. In the upstream region of the FHA open reading frame, a promoter-like element was found containing sequences homologous to *E. coli*-10 and -35 promoter boxes. However, the distance between the two boxes is too long (22 nucleotides) to function efficiently in *E. coli*. This long distance is reminiscent of the promoter sequence found in the PT gene (Fig. 5).

FHA

TTGACT AGAAATTTCCTACAAGTCTTG **TATAAA**

-35 22 **-10**

PTX

CTGACC CCCCTGCCATGGTGTGATCCG **TAAAAT**

-35 21 **-10**

E. coli

TTGACA NNNNNNNNNNNNNNNNN **TATAAT**

-35 17 **-10**

FIG. 5. Comparison of proposed promoter structures of the genes coding for PT and FHA with *E. coli* promoters. The proposed -35 and -10 boxes of the FHA, PT, and *E. coli* promoters are indicated in bold. Distances between the two boxes are indicated by the numbers of nucleotides found in the FHA, PT, and *E. coli* promoters. N designates any nucleotide.

Following the promoter-like sequence, three possible ATG initiation codons were found in frame with the FHA open reading frame. Computer analysis suggests that the first of these three codons may be the initiation codon. Although FHA is a secreted protein, no typical signal peptide follows any of the three possible initiation Methionines. Rather, a relatively long hydrophilic region containing both basic and acidic amino acids was found to precede a hydrophobic region that possibly acts as a membrane-spanning domain. Work is in progress to assess whether this unusual structure may serve as a signal peptide.

Next is a region of over 100 amino acids that features about 50% sequence identity with the amino-terminal parts of calcium-independent hemolysins of *Serratia marcescens* (48) and *Proteus mirabilis* (60). The homology to the *P. mirabilis* hemolysin is particularly significant since the latter's DNA has a very different GC content from that of the *B. pertussis* DNA. The structure-function relationship of the *S. marcescens* hemolysin has been studied in some detail; Schiebel and Braun (53) recently showed that its amino-terminal region is involved in binding to and insertion into erythrocytes. Based on the sequence similarity in that region with the FHA protein, it is tempting to speculate that FHA's amino-terminal portion is also involved in binding the molecule to its receptors. A site responsible for binding to integrin CR3 on macrophages has been identified in the middle portion of the FHA protein; the site includes a functional RGD site (50). However, FHA interacts with two classes of molecules (59), and the amino-terminal homologous region could constitute a second, carbohydrate-binding site of the protein.

Secretion and activation of the calcium-independent hemolysins is dependent on a second gene. Deletion analysis of the hemolysin structural gene has suggested that the product of this second gene interacts with the amino-terminal homologous region of the hemolysin protein during its secretion and activation steps (54). FHA is also a secreted protein whose secretion is very efficient since analysis by SDS-PAGE of the culture medium indi-cated that FHA is by far the major protein secreted by *B. pertussis*. If the homologous region is indeed involved in FHA secretion, its mechanism is likely to be very similar to the hemolysin secretion mechanism. Other genes therefore may be involved in this step. Currently this possibility is being investigated by analyzing genes different from the FHA structural gene that, when inactivated by transposon insertions, result in an FHA$^-$ phenotype.

The homologous region is followed by regions containing direct repeats. These repeats have no sequence similarity with any other protein whose sequence is known so far. They also do not share characteristics with repeats found in other filamentous proteins. The role of these repeats therefore remains to be investigated.

FHA secreted by *B. pertussis* is a 220-kDa protein, whereas the FHA open reading frame has a coding capacity for a 370-kDa protein. Post-translational cleavage steps are thus likely to occur during or after synthesis and/or secretion. Several portions of the FHA open reading frame have been expressed in *E. coli* and probed with a variety of antibodies, thereby allowing FHA to be subdivided into three immunological domains. The amino-terminal third of the protein, including the domains homologous to the hemolysins and direct repeats, does react with polyclonal antisera raised against FHA purified from *B. pertussis.* This finding confirms that the amino-terminal region is part of the secreted portion of FHA.

The middle portion of the FHA, including the RGD sequence, is highly immunogenic. Polyclonal antisera as well as all of 14 monoclonal antibodies were reactive to that portion. Using monoclonal antibodies, at least four different epitopes could be identified in that region. This finding favors the notion that the middle portion is highly exposed, consistent with the presence of an exposed integrin-binding site. The antibodies reactive to that region are now being tested for their binding-inhibitory effects as well as their protective potential in animal models.

Finally, the carboxy-terminal third of the molecule did not react with any of the monoclonal antibodies or the polyclonal antisera against purified FHA. This region may therefore be the portion that is cleaved during or after production and secretion of the mature FHA. Polyclonal antisera against whole *B. pertussis* organisms were also developed to investigate whether this carboxy-terminal region remained cell associated and would therefore be recognized by antibodies against whole organisms. Although high expression levels were obtained from at least some of these 3′ fragments, no reactivity was observed with such antibodies, suggesting that this region is either immunosilent or that it degraded too rapidly in the *B. pertussis* cultures to be recognized by the immune system. Fig. 6 shows a model of the FHA structure, based on the above analysis.

With the current debate over the safety of pertussis vaccines, it is desirable to investigate the structure-function relationship of the important antigens to be included

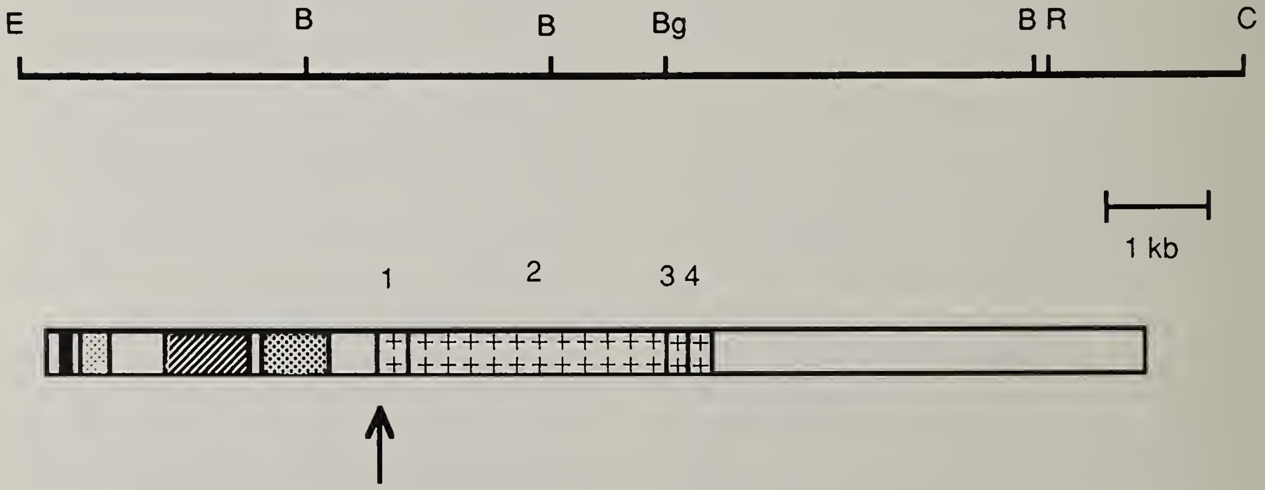

FIG. 6. Proposed structure of FHA and its structural gene. The top line represents a restriction map of the FHA structural gene. B, BamH1, Bg, Bgl2, C, Cla1, and R, EcoR1. The following striking features of the FHA open reading frame are indicated below the restriction map: the ■, a possible hydrophobic transmembrane domain; ░, the portion of FHA that shares sequence similarity with the hemolysins of *S. marcescens* and *P. mirabilis;* ▨ and ▒, regions containing amino acid repeats; ⊞, the most immunogenic portion of the FHA protein containing at least four different epitopes, (indicated by the numbers 1 to 4) recognized by monoclonal antibodies; ↑, a proteolytic cleavage site.

in future acellular vaccines. The ultimate goal would be to include in these vaccines only those structures that provide protective immunity and eliminate those that possibly may induce undesirable side effects. Studies such as the one presented herein may help provide a rational strategy for developing such new-generation, safer vaccines. Studies on PT may lead to fully detoxified PT-CRM, and studies on FHA may provide knowledge of the nature of its protective epitopes.

ACKNOWLEDGMENTS

This project was supported by the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture and the Administration des Technologies Nouvelles de la Région Wallonne.

This work would have been impossible without the help of R. Antoine, A.-M. Delisse-Gathoye, G. Dequesne, and C. Feron. We also thank B. Colau for oligonucleotides, M. Francotte, I. Heron, and E. Simoens for antibodies, and J. J. Mekalanos and S. Stibitz for plasmids.

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DISCUSSION

DR. HANSKI: I would like to ask you about the deletion of the C terminal, if it had the same effect with respect to all G proteins, namely, Gi, Gs and the same question about the Gt.

DR. LOCHT: The only ones we have looked at so far is the G proteins present in CHO cells, and I think that is Gi. And for quantitative measurements, transducin or the Gt protein. It is very difficult to purify enough quantities of the other G proteins to really have a feel for the degree of decrease of enzymatic activity. But in two cases, the CHO cell Gi proteins and the transducin gave similar results.

DR. SHAHIN: Can you tell me if your method for immunization of mice to prepare monoclonal antibodies, used pure FHA as the immunogen?

DR. LOCHT: Yes, I did not do this work. It was done in Copenhagen and if somebody can correct me, I think it was done using purified FHA in Freund injected IP with one or two boosters.

DR. SHAHIN: I was just wondering if there could be a technical explanation for the fact that your monoclonals all bind in the middle and if you just made more monoclonals you could find some of that bind to the amino terminal.

DR. LOCHT: Yes, we have looked at 14 different monoclonals but there are more in the works and we are looking at them.

DR. PARKER: I could say that we have used both whole cells and outer membrane proteins to generate anti-FHA antibodies and they all react within that same region that Camille was talking about. We have tested six independent ones.

DR. LEININGER: I am not sure where that 90k fragment is. The typical degradation of FHA as I see on gels is 220, the mature protein, 150 and I see a 98 and then with time you can get further breakdown. Is that 90, that 98 that I am seeing or is that something else? And have you done amino acid sequence on that 90 fragment to localize it there or is it just based on your antibody binding of your fragments?

DR. LOCHT: The bands that we usually see using purified material from SmithKline, there are usually four bands, actually, one at 220, a doublet at 150, 140, and one band that is between 90 or 95 but sometimes not quite that clear. When the amino acid sequences were attempted, they were unsuccessful in the 90, 95 kD fragment so we could get amino acid sequences of all the other peptides and they are surprisingly identical. So I think it is actually proteolytic cleavage that we actually look at but from the 90 it was not successful. So the mapping was done using the monoclonals.

DR. COWELL: An alternative explanation for your C terminal hydrophobic region is that it is not directly involved in G binding protein but it is involved in confirmation. Unless you have other data I would suggest that that is a likely explanation and the reason I say that is we work with CRM 3201 as you know, and Bill Black made that in Stan Falkow's laboratory and we worked with it and that is a four amino acid insertion in and around amino acid 106 or 107. You have data that doing site-directed mutagenesis around that region does not affect things very much, but what we find is that when that purified CRM is analyzed, it is reduced to 60 percent of any deglycohydrolase. It has only twenty percent of the ADP levels of ribosyltransferase, but interestingly, it only has about two to three percent of any biological activity. So my interpretation of that is that we are affecting the structure of the S-1 and the confirmation so it is reducing a number of biological activities and even differences in NAD and ADP ribosyltransferase.

DR. LOCHT: Well, when we did kinetic measurements on any deglycohydrolase activity of the truncated molecules versus the full length one, we did not see any difference. Second of all, when we used a number of monoclonals, I think we have used about 50 different ones that were known to react against confirmational epitopes, there was no difference. Third argument is, and I refer to the poster by Joe Barbieri and Cortina, they now have data to show that the difference is a Km difference so it looks like it is actually a G protein binding site, the C terminus.

DR. ULLMANN: Do your antibodies cross-react with the hemolysins and if not, what is your interpretation of the significance of this homology?

DR. LOCHT: First of all we have not looked at the monoclonals that we have for cross-activity because they react to a different region as compared to the homologous region. Second of all, I have been talking to Rod Welsh who has also sequenced the protein, the hemolysin that is calcium-independent and also contains the same region of homology and he promised me to send some antibodies so we can look at that.

DR. SAUKKONEN: We have recently suggested that the sub-units of pertussis toxin S-2 and S-3 mediate the binding to ciliated cells and macrophages so that S-2 would be responsible for mediation to cilia and S-3 to macrophages. We wanted to determine the importance of each of these molecules. S-2 and S-3 share a significant homology with the eukaryotic carbohydrate recognition domain, CRD. With site directed mutations of certain areas of these regions, for example, this area around the cysteine 91, we could abolish all the binding to both cilia and macrophages. We suggest that this area is critical to the overall structure of the CRD.

Both S-2 and S-3 contain this region (inaudible)... they both contain this region which is also in the eukaryotic CRD, Mutating this area, we could change the binding specificity to certain carbohydrates. I will present the detailed map of this region and binding experiments in the poster session. This region appears to be crucial, in so far, as if you take this region away from S-2 and replace it with the corresponding region from S-3, you are going to change the binding of S-2 to cilia to be like the binding of S-3 to macrophaages.

Pertussis Toxin and Its Mode of Entry into Eukaryotic Cells

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Pertussis toxin is a typical bacterial toxin in that it is composed of an enzymatically active A subunit and a binding component known as the B oligomer (30). The A, or S1, subunit is a single polypeptide chain (M_r = 26,000) which has ADP-ribosyltransferase activity (19, 23). The cellular substrates for this toxin belong to a family of GTP-binding regulatory proteins (G proteins) which are involved in eukaryotic cell signal transduction (4, 18). These G proteins normally transmit signals through the plasma membrane of the cell. When the G proteins are ADP-ribosylated by pertussis toxin, this function is abolished, and the cell no longer responds to a variety of hormones and neurotransmitters (10).

The B oligomer of pertussis toxin, which contains the binding site for the eukaryotic receptor, is made up of five subunits, i.e., one molecule each of S2, S3, and S5, and two molecules of S4; these assemble into an oligomeric structure (30). The pertussis toxin receptor on Chinese hamster ovary (CHO) cells appears to be a glycoprotein since variant CHO cells which lack terminal NeuAc→Galβ4GlcNAc residues on glycoproteins were resistant to toxin action (3). Subsequent studies have demonstrated that variant CHO cells which are deficient in sialic acid residues on cell surface macromolecules (both glycoproteins and glycolipids) are also resistant to pertussis toxin action, suggesting that sialic acid residues may be important carbohydrate residues on the CHO cell receptor (32).

While research has elucidated the nature of the pertussis toxin receptor on CHO cells, little is known about the events that take place after the toxin binds to the eukaryotic cell surface. Substrates for pertussis toxin are associated with the plasma membrane and are assumed to be located on its inner face (10). Therefore, the A subunit must somehow enter into or through the plasma membrane in order to ADP-ribosylate its cellular target. This study examined the mechanism by which pertussis toxin enters the eukaryotic cell and compared its entry to that of other ADP-ribosylating toxins.

MATERIALS AND METHODS

Materials. Pertussis toxin was obtained from the Michigan Department of Public Health (Lansing, Mich.). Cholera toxin was purchased from Sigma Chemical Company (St. Louis, Mo.). Diphtheria toxin was the generous gift of Dr. William Habig (Center for Biologics Evaluation and Research, Bethesda, Md.).

Cytotoxicity assays. CHO cell clustering and elongation were determined 24 h after exposure of the cells to either pertussis toxin or cholera toxin, as previously described (12, 13). Cells were visually examined 48 h after their exposure to determine cell death due to diphtheria toxin.

ADP-ribosylation assays. The ability of pertussis toxin or diphtheria toxin to ADP-ribosylate their cellular substrates was determined by exposing the cells to toxin, harvesting membranes (for pertussis toxin) or cytosol (for diphtheria toxin), and subsequently exposing the membranes or cytosol to more toxin in the presence of [32P]NAD, as previously described (32).

RESULTS AND DISCUSSION

In general, bacterial toxins are thought to enter eukaryotic cells either by endocytosis or direct penetration of the plasma membrane (Fig. 1). Diphtheria toxin is an example of a toxin that enters the cell by endocytosis (27); cholera toxin is currently thought to directly enter the cell through the plasma membrane (8). Like pertussis toxin, both diphtheria toxin and cholera toxin are ADP-ribosylating toxins. Diphtheria toxin modifies elongation factor 2 (EF-2), a cytoplasmic protein essential for protein synthesis. ADP-ribosylation of EF-2 inactivates the protein, resulting in a shutdown of protein synthesis and eventual cell death (5). Cholera toxin modifies a G protein, termed G_s, which is found on the inner leaflet of

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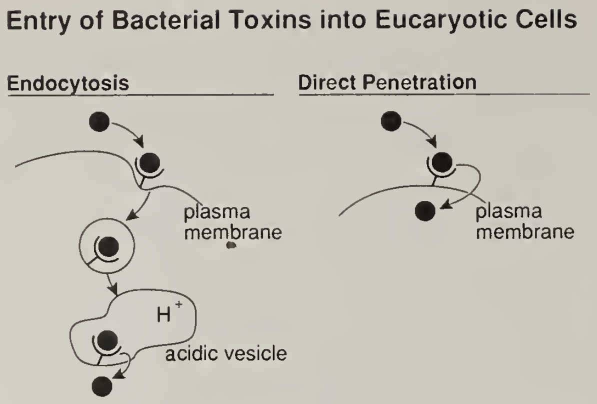

FIG. 1. Potential pathways for entry of bacterial toxins into eukaryotic cells.

the plasma membrane (10) and which is distinct from the G proteins that are pertussis toxin substrates. G_s regulates adenylate cyclase; it has also been implicated in the regulation of certain ion channels (29, 33). ADP-ribosylation of G_s by cholera toxin converts the protein to a form which continually stimulates adenylate cyclase, thus raising the cAMP levels of the cell.

The mechanism of diphtheria toxin endocytosis and the toxin's subsequent entry into the cytoplasm has been extensively studied. Diphtheria toxin binds to receptors on the cell surface which are thought to be glycoproteins (15). The toxin is endocytosed and ultimately enters acidic intracellular vesicles known as endosomes (27). The pH of these vesicles appears to be critical for toxin entry since agents such as NH_4Cl which raise the pH of endosomes will protect cells from diphtheria toxin action (2, 16, 20, 21). Diphtheria toxin has been shown to undergo a conformational change at low pH; also, this more hydrophobic form of the molecule may penetrate the membrane in a way that allows the toxin's enzymatically active fragment to be delivered into the cytosol where it ADP-ribosylates EF-2 (1, 7).

The mechanism of cholera toxin entry into the cell is less well-defined. Cholera toxin is composed of six subunits, i.e., one copy of the A, or enzymatically active, subunit and five copies of the B protomer (9). The B protomers assemble to form a pentameric structure known as the B subunit which binds to the eukaryotic cell receptor. The cholera toxin receptor has been identified as ganglioside G_{M1} (6, 14). It has been postulated that the toxin's binding to receptor induces a conformational change in the toxin molecule that allows hydrophobic portions of the A subunit to interact with the plasma membrane. This interaction in turn results in subsequent penetration of the A subunit into or through the plasma membrane where it ADP-ribosylates G_s (8, 26). Other evidence indicates, however, that cholera toxin may enter at least certain cell types by endocytosis (17, 22).

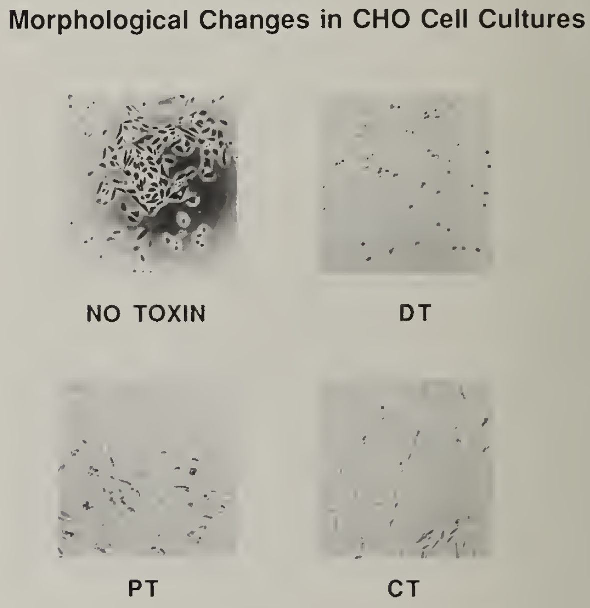

FIG. 2. Morphological changes in CHO cells induced by toxins. CHO cells were cultured in 96-well microtiter plates and were exposed to either no toxin, pertussis toxin (10 ng/ml), diptheria toxin (60 ng/ml), or cholera toxin (1.6 ng/ml) as indicated. The cells are shown 24 h (pertussis toxin and cholera toxin) or 48 h (diptheria toxin) after addition of toxin.

To begin defining the mechanism by which pertussis toxin enters eukaryotic cells, this study compared the sensitivities of pertussis toxin, cholera toxin, and diphtheria toxin to various agents that alter entry of bacterial toxins. Because CHO cells exhibit unique morphological changes upon intoxication with either pertussis toxin, cholera toxin, or diphtheria toxin (Fig. 2), they were used as the target cells for the toxins in this study. CHO cell exposure to pertussis toxin cells results in a clustered appearance of the cells (13); the cells elongate when exposed to cholera toxin due to increased intracellular cAMP levels (12). Finally, diphtheria toxin causes CHO cell death by inhibiting protein synthesis (25). These changes in cell morphology were used as assays for toxin action.

CHO cell sensitivity to diphtheria toxin was markedly inhibited by NH_4Cl, as has been reported previously for other cell types (2, 16, 20, 21). In contrast, cholera toxin action was unaffected by this agent. Pertussis toxin behaved like cholera toxin in that the ability of the pertussis toxin to cluster CHO cells was unaffected by NH_4Cl (S. Hausman and D. Burns, manuscript in preparation).

The ability of cytochalasin B to affect the action of pertussis toxin and diphtheria toxin was examined. Cytochalasins inhibit endocytosis of ricin in Vero cells (28) and intoxication of CHO cells by *Bacillus anthracis* adenylate cyclase toxin (11). The mechanism by which cytochalasins, compounds which disrupt microfilament structure (31), inhibit endocytosis remains unknown. The study found that whereas the ability of diphtheria toxin to ADP-ribosylate EF-2 was decreased when cells were pretreated with cytochalasin B, this agent had no effect on pertussis toxin action.

These results suggest that pertussis toxin enters CHO cells by a mechanism distinct from that used by diphtheria toxin. Since NH_4Cl had no effect on pertussis toxin action, pertussis toxin unlike diphtheria toxin, does not require an acidic environment for entry into the host cell. The finding that cytochalasin B does not affect pertussis toxin entry suggests that this toxin may not enter the cell by an endocytic pathway. However, because cytochalasins may not block all types of endocytosis in all cell types (28), an endocytic pathway for pertussis toxin entry cannot be completely excluded based on these data. The data are also consistent with the idea that pertussis toxin may penetrate the plasma membrane of CHO cells directly. Montecucco et al. have shown that pertussis toxin can interact directly with detergent micelles (24). This finding suggests that after pertussis toxin binds to its receptor, the toxin may interact directly with the lipids in the outer leaflet of the plasma membrane. The elucidation of the exact mechanism of pertussis toxin entry requires further study.

ACKNOWLEDGMENTS

This work was supported in part by Participating Agency Support Agreement BST-5947-P-HI-4265 between the Agency for International Development and the Public Health Service.

We thank Jane Halpern for helpful discussions.

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DISCUSSION

DR. GOLDMAN: Has anyone done studies looking at accumulation of receptors clustering or accumulation in clathrin coated pits with pertussis toxin binding?

DR. BURNS: With pertussis toxin, not to my knowledge.

DR. ASKELÖF: For a number of years now I have been claiming that the CHO cell assay is a binding assay and not an enzyme assay. I think your data could support that. What do you think?

DR. BURNS: I think that the data that Dr. Rappuoli showed and work we have done with Neil Burnett would indicate that the CHO cell assay actually represents something that happens after the G protein is ADP ribosylated. ADP ribosylation is absolutely necessary for this clustering.

DR. ASKELÖF: Yes, but you know, of course, that one of the reasons why I say this is that by use of a synthetic peptide from pertussis toxin, we can induce CHO cell clustering which is indistinguishable from that induced by pertussis toxin.

DR. BURNS: I think we would all be interested in seeing that data.

Pertussis Toxin Substrates: Characterization of Multiple Forms of $G_{o\alpha}$ mRNA and the Requirements for Toxin-Catalyzed ADP-Ribosylation of $G_{o\alpha}$ and $G_{t\alpha}$

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The effects of some hormones, neurotransmitters, and drugs are exerted through membrane-associated receptors coupled to intracellular effectors through guanine nucleotide-binding (G) proteins (2, 30). The best defined examples of signal-transducing pathways utilizing G proteins are the adenylyl cyclase (1, 10) and visual excitation complexes (33); in some cells, G proteins also regulate phospholipid turnover (7) and ion channels (2). G proteins are composed of α, β, and γ subunits; the α subunits are responsible for guanine nucleotide-binding and GTP hydrolysis (1). Activation of the G protein results from binding GTP to α and the dissociation of α(GTP) from βγ; inactivation is the result of GTP hydrolysis to GDP and reassociation of subunits (1, 10). Agonist-occupied receptors promote activation in part by facilitating GDP release, thereby promoting GTP binding (1, 2, 10).

Pertussis toxin (PT) affects animal cells by ADP-ribosylating the α subunits of susceptible G proteins (23, 36). ADP ribosylation uncouples G proteins from their receptors, blocking signal transduction. The pleiotropic effects of PT on animal cells result from its ability to ADP ribosylate G proteins involved in a variety of regulatory pathways. G_o, a G protein found primarily in brain, has been shown to couple in vitro to a number of receptors (e.g., muscarinic cholinergic, dopamine, light) and may participate in the regulation of calcium channels (31). Transducin (G_t) is found in rod outer segments and participates in signaling from the photon receptor rhodopsin to its effector, a cyclic GMP phosphodiesterase (33).

$G_{o\alpha}$ was initially identified in bovine brain (25, 32). Recent immunological and structural studies have demonstrated that there are in fact two related forms of $G_{o\alpha}$, termed $G_{o\alpha1}$ and $G_{o\alpha2}$ (16, 22, 31). Deduced amino acid sequences obtained from cDNA clones corresponding to $G_{o\alpha1}$ and $G_{o\alpha2}$ indicate that the two proteins have identical sequences in the amino-terminal two-thirds of the coding region (16). The carboxy termini are, however, highly related but clearly different. Based on the finding that there is one human gene for $G_{o\alpha}$ located on chromosome 16 (J. J. Murtagh, R. Eddy, T. B. Shows, J. Moss, and M. Vaughan, J. Cell Biol. 107:4015, 709a), it appears that the different forms of $G_{o\alpha}$ arise by alternative splicing.

The carboxy termini of PT-sensitive G_α subunits are highly conserved (27). Perhaps because of this conservation these G proteins are capable of interacting in vitro with more than one receptor. For example, photolyzed rhodopsin stimulates GTP hydrolysis by G_t, G_o, and G_i (3, 4, 9, 17, 35). In addition to interaction with the receptor, the carboxy terminus of G_α subunits is involved in the interaction with the γ subunit of $G_{\beta\gamma}$ and the amino terminus, which has been thought to be involved in the binding of $G_{\beta\gamma}$ (26), is in fact located in proximity to the carboxy terminus (14, 15, 37). The carboxy terminus, which is the target of PT-catalyzed ADP-ribosylation (40), may thus be critical for G_{α} action. PT clearly exhibits considerable specificity in selectively catalyzing the ADP-ribosylation of the Cysteine residue near the carboxy terminus (40). Since the carboxy end of $G_{o\alpha}$ is altered by splicing and it is also involved in interaction with receptors, one effect of splicing alternative carboxy termini may be to modulate the receptor specificity.

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This report examines the role of the carboxy terminus of G proteins. To determine the role of $\beta\gamma$ in ADP-ribosylation, a monoclonal antibody was used that recognizes $G_{t\alpha}$ at a region critical to $G_{\beta\gamma}$ interaction; a second monoclonal antibody that recognizes $G_{t\gamma}$ was used to determine its possible functions in ADP-ribosylation. To determine the extent to which the amino acids in the vicinity of the Cysteine contribute to its recognition as a substrate by PT, in vitro mutagenesis of $G_{o\alpha}$ was undertaken. Since the carboxy end of $G_{o\alpha}$ is important for receptor recognition and since different $G_{o\alpha}$ mRNAs yield differences in carboxy termini, the research focused on the expression of these different forms of $G_{o\alpha}$ mRNA. In these studies, $G_{o\alpha}$-specific oligonucleotides were used to differentiate among G_o and related G protein α subunit mRNAs as well as among different forms of $G_{o\alpha}$ mRNA.

Abbreviations. The following abbreviations are used: G proteins, the family of heterotrimeric GTP-binding proteins composed of α (39 to 52 kDa), β (35 to 36 kDa), and γ (8 to 10 kDa) subunits; G_i, the subfamily of inhibitory G proteins whose α subunits, $G_{i\alpha1}$ (41 kDa), $G_{i\alpha2}$ (40 kDa), and $G_{i\alpha3}$ (40 to 41 kDa) are ADP-ribosylated by PT; G_t, the transducins whose α subunits $G_{t\alpha1}$ (39 kDa) and $G_{t\alpha2}$ (41 kDa) are found in rods and cones, respectively, and are ADP-ribosylated by PT; G_o, the "other" G protein found primarily in brain and neural tissues whose α subunits $G_{o\alpha1}$ and $G_{o\alpha2}$ (both $\propto$39 kDa) are ADP- ribosylated by PT; $G_{t\beta\gamma}$, the transducin β, γ complex; and $G_{t\gamma}$, the transducin γ subunit.

MATERIALS AND METHODS

[α–^{32}P]dATP (6,000 Ci/mmol) was purchased from New England Nuclear Corp.; terminal deoxynucleotidyltransferase and RNA standards from Bethesda Research Laboratories, Inc., and nylon membrane (Nytran) from Schleicher & Schuell, Inc. Other materials are as noted in the referenced publications or in figure or table legends.

Oligonucleotide synthesis. Oligonucleotides were synthesized by Pharmacia or within our laboratory using an Applied Biosystems 380B DNA synthesizer. Sequences of oligonucleotides used for Northern blot hybridization are as follows: (i) GoC, a 48-base oligonucleotide probe specific for the coding region common to $G_{o\alpha1}$ and $G_{o\alpha2}$, 5′ GAA GGG CTC CGT GTC TTC CAT CCG ACT CAC CAC GTC ACA CAC CAT CTT 3′ (complementary to bases 313 to 360 of a bovine $G_{o\alpha1}$ cDNA clone λG09 [38]); (ii) 5′C, 31-base oligonucleotide probes specific for the 5′-untranslated region of human or bovine $G_{o\alpha}$, 5′ GAA ACA AAA ATG TCG GGA AAC CAG AAC CCT C 3′ (complementary to bases −163 to −133 of a human genomic clone for $G_{o\alpha}$ [20]) and 5′ AAA ACA AAA ATG TCG AGA AAC CAG AAC CCC C 3′ (complementary to bases −162 to −132 of a bovine $G_{o\alpha1}$ cDNA clone λG09 [38]); (iii) Go1, a 36-base oligonucleotide probe specific for $G_{o\alpha1}$ coding region, 5′ TTT GTT GGG TGA GCG GTT TTT GCT TTC AAA CTG TGT 3′ (complementary to bases 916 to 951 of a rat $G_{o\alpha1}$ cDNA clone [16]); (iv) Go2, a 36-base oligonucleotide probe specific for $G_{o\alpha2}$ coding region, 5′ CTT GTG AGC TGA TTT GTT CTT ACT CTC ATA CTG CCC 3′ (complementary to bases 916 to 951 of a hamster $G_{o\alpha2}$ cDNA clone [16]); (v) 3′A, a 48-base oligonucleotide probe specific for $G_{o\alpha1}$ 3′-untranslated region A, 5′ AGG CTG TGT GTC TTA ACA AAG GCC AAA AGG TCA TGC TAC CAG GAG ATC 3′ (complementary to bases 60 to 107 of the 3′-untranslated region of bovine $G_{o\alpha1}$ clone λG09 as described by Price et al. [28]); and (vi) 3′B, 48-base oligonucleotide probes specific for the human or bovine $G_{o\alpha1}$ 3′-untranslated region B, 5′ TTT GTT ATG CCT CTT TAA AGT TTA TTT ACC AAC TTG CAT TTG TTA GTG 3′ (complementary to bases 1 to 48 following the variable number of tandem repeats which start at base 76 of the human 3′-untranslated region B (J. J. Murtagh, J. Moss and M. Vaughan, unpublished data) and 5′ TTT GTT ATG CCT TTT TGG AAT TTG TTT ACC AAC TTG CAT TTG TTA GTG 3′ (complementary to bases 92 to 139 of the 3′-untranslated region of bovine $G_{o\alpha1}$ clone λG03.1 as described by Price et al. [28]).

Preparation of RNA. Total cellular RNA was isolated from either human neuroblastoma cells (IMR–32) or bovine brain and retina (5), and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography.

Poly(A)$^+$ RNA (5 μg) was fractionated by electrophoresis in a 1.2% agarose/2.2 M formaldehyde gel and transferred to nylon membrane. Two different blots, I and II, were prepared. Prehybridizations were performed at 42°C for 6 h in 5x SSC/5x Denhardt's solution (1x = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/10 mM Tris Cl$^-$, pH 7.4/40% formamide/0.1% sodium dodecyl sulfate/10% dextran sulfate containing denatured salmon sperm DNA (100 µg/ml) followed by hybridization in the same solution at 42°C for 16 h with 48-base oligonucleotides, labeled with [α–^{32}P]dATP using terminal deoxynucleotidyltransferase (8). Hybridizations with other oligonucleotide probes were performed as described above except that formamide was omitted from the hybridization solution. Filters were washed once at 55°C with 2x SSC/0.5% sodium dodecyl sulfate and twice with 0.5x SSC/0.5% sodium dodecyl sulfate. Filters were exposed to Kodak XAR film at –80°C with an intensifying screen.

RESULTS

Identification of multiple $G_{o\alpha}$ mRNAs. Earlier studies demonstrated that $G_{o\alpha}$ is primarily present in brain, retina, and related tissues (29, 31). Examination of $G_{o\alpha}$ mRNAs by hybridization of bovine brain poly(A)$^+$ RNA with an oligonuc-leotide probe specific for the $G_{o\alpha}$ coding region (probe GoC, which was designed to discriminate between $G_{o\alpha}$ mRNA and related G_α mRNAs) revealed a complex banding pattern (Fig. 1). Hybridizing bands were detected at 6.2, 4, 3, and 2 kb (Fig. 1). To determine the basis for this complexity, oligonucleotide probes were designed based on known cDNA sequences that were specific for $G_{o\alpha}$ 5′– and 3′–untranslated regions and the alternative carboxy termini of the coding region.

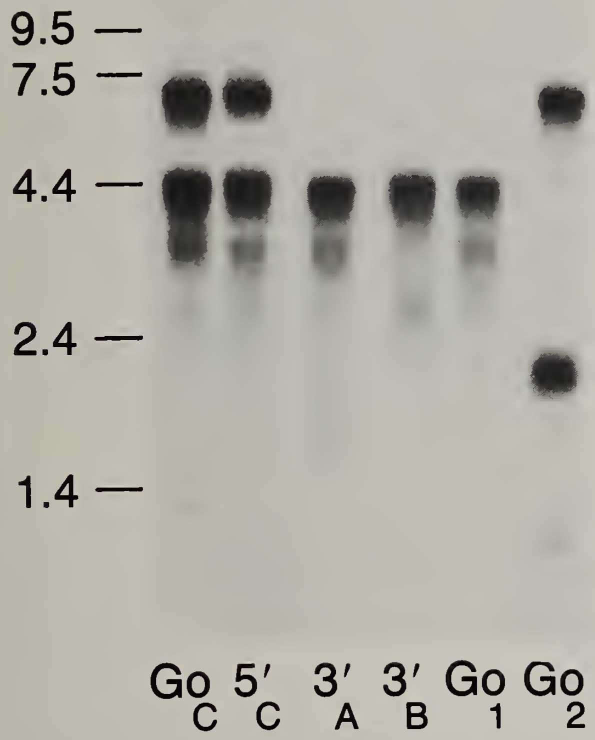

FIG. 1. Hybridization of bovine brain poly(A)$^+$ RNA with $G_{o\alpha}$-specific oligonucleotide probes. Poly(A)$^+$ RNA from bovine brain was hybridized with probes specific for the $G_{o\alpha}$ coding region common to $G_{o\alpha1}$ and $G_{o\alpha2}$ (GoC), bovine 5′-untranslated region (5′C), 3′-untranslated region A, (3′A), bovine 3′-untranslated region B (3′B), $G_{o\alpha1}$ coding region (Go1), and $G_{o\alpha2}$ coding region (Go2). GoC, 5′C, 3′A and 3′B were hybridized with blot I; Go1 and Go2 were hybridized with blot II as described in text.

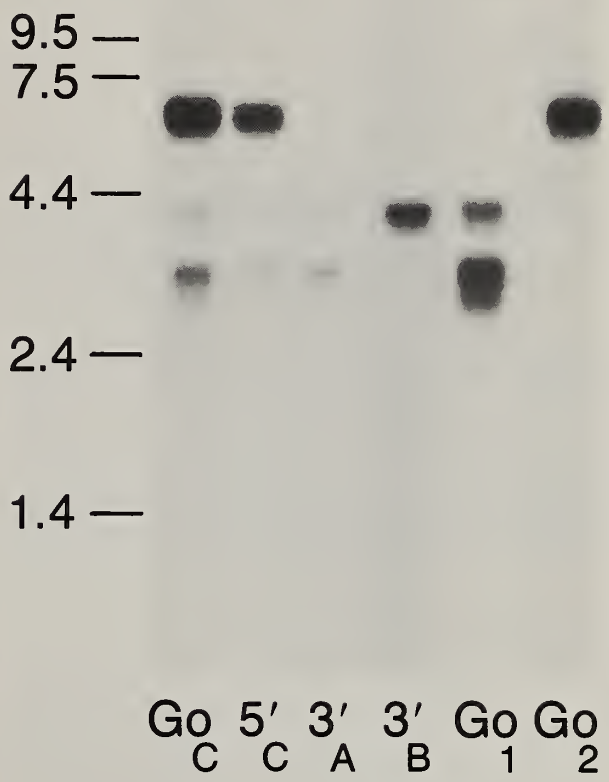

FIG. 2. Hybridization of human neuroblastoma poly(A)$^+$ RNA with $G_{o\alpha}$-specific oligonucleotide probes. Poly(A)$^+$ RNA from human neuroblastoma cells (IMR-32) was hybridized with probes specific for the $G_{o\alpha}$ coding region common to $G_{o\alpha1}$ and $G_{o\alpha2}$ (GoC), human 5′-untranslated region of $G_{o\alpha}$ (5′C), 3′-untranslated region A (3′A), human 3′-untranslated region B (3′B), $G_{o\alpha1}$ coding region (Go1), and $G_{o\alpha2}$ coding region (Go2). GoC, 3′A and 3′B were hybridized with blot I; 5′C, Go1 and Go2 were hybridized with blot II.

Hybridization of bovine brain poly(A)$^+$ RNA with a probe to the 5′–untranslated region detected all four major bands (Fig. 1). These bands were subclassified based on their ability to hybridize with two probes for the $G_{o\alpha 1}$ 3′–untranslated region. Probe 3′–A detected bands at 4 and 3 kb, whereas probe 3′–B detected the bands at 4 and 2 kb (Fig. 1) (28). The 6.2–kb band was not detected by either probe 3′–A or 3′–B (Fig. 1). Hybridization of poly(A)$^+$ RNA with probe Go1 detected primarily the major bands at 4, 3, and 2 kb (Fig. 1). Probe Go2 detected primarily the 6.2–kb band; a band at ∝1 kb was also observed. Based on its size and the fact that it does not hybridize with other $G_{o\alpha}$ probes, this latter band does not appear to be a $G_{o\alpha}$–related mRNA.

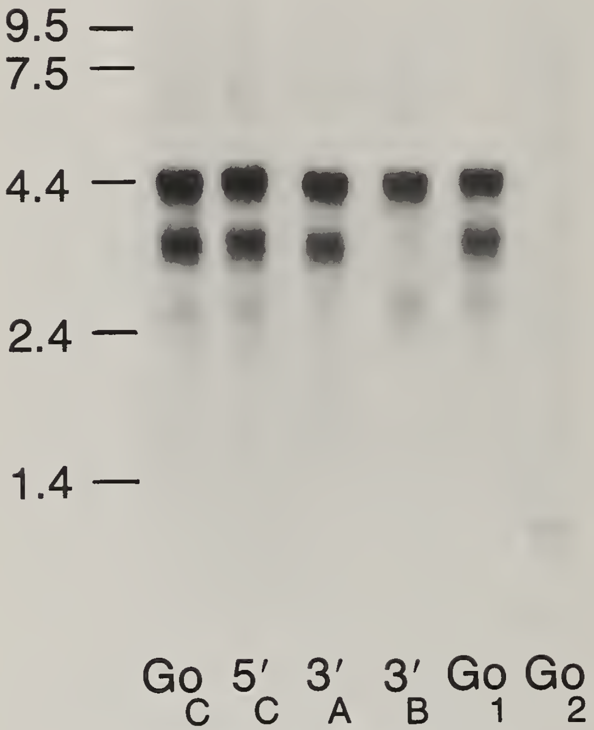

FIG. 3. Hybridization of bovine retina poly(A)$^+$ RNA with $G_{o\alpha}$-specific oligonucleotide probes. Bovine retinal poly(A)$^+$ RNA was hybridized with probes specific for the $G_{o\alpha}$ coding region common to $G_{o\alpha 1}$ and $G_{o\alpha 2}$ (GoC), bovine 5′-untranslated region (5′C), 3′-untranslated region A (3′A), bovine 3′-untranslated region B (3′B), $G_{o\alpha 1}$ coding region (Go1), and $G_{o\alpha 2}$ coding region (Go2). GoC, 5′C, 3′A, and 3′B were hybridized with blot I; Go1 and Go2 were hybridized with blot II.

Hybridization of poly(A)$^+$ RNA from human neuroblastoma cells with the same array of probes gave a different banding pattern, suggesting that the alternative expression of $G_{o\alpha}$ mRNA may be cell-specific (Fig. 2). The neuroblastoma cells did synthesize mRNA specific for both $G_{o\alpha 1}$ and $G_{o\alpha 2}$ (Fig. 2). Bovine retinal poly(A)$^+$ RNA had a different banding pattern than that seen with the other samples, but, again, both forms of $G_{o\alpha}$ mRNA were observed (Fig. 3).

Immunological characterization of $G_{t\alpha}$. A monoclonal antibody (MSN1) directed against $G_{t\alpha}$ inhibited light-activated rhodopsin-stimulated GTP hydrolysis (data not shown). MSN1 also inhibited PT-catalyzed ADP-ribosylation of $G_{t\alpha}$ in the presence of $G_{t\beta\gamma}$ (Fig. 4). Proteolysis by trypsin of the amino terminal 2 kDa of $G_{t\alpha}$ abolished immunoreactivity (data not shown); these data are compatible with the participation of the $G_{t\alpha}$ amino terminus in the MSN1 epitope. As might be expected, based on data supporting the binding of $G_{t\beta\gamma}$ to the amino terminus of $G_{t\alpha}$, $G_{t\beta\gamma}$ inhibited MSN1 reactivity with $G_{t\alpha}$ (Table 1).

Light-activated but not dark rhodopsin also inhibited antibody reactivity (Table 1). In the presence of both rhodopsin and $G_{t\beta\gamma}$, inhibition of immunoreactivity appeared to be synergistic (Table 1). Since rhodopsin is thought to interact with the carboxy terminus, its inhibi-

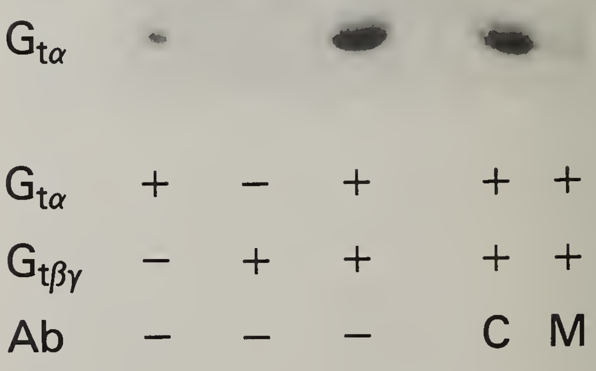

FIG. 4. Monoclonal antibody MSN1 inhibits PT-catalyzed ADP-ribosylation of $G_{t\alpha}$. $G_{t\alpha}$ (2 µg), $G_{t\beta\gamma}$ (3 µg), and PT (2.5 µg) were incubated with MSN1 or control immunoglobulin G for 45 min at 4°C before initiating PT-catalyzed ADP-ribosylation. ADP-ribosylation was carried out at 30°C in a final volume of 0.15 ml with 50 mM potassium phosphate (pH 7.5), 2.5 µM NAD, 1 mM ATP, and 2 µCi of [α-^{32}P]NAD. Trichloroacetic acid-precipitated proteins were subjected to electrophoresis in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, followed by autoradiography with Kodak XAR film and an intensifying screen at -70°C for 1 h. M, Monoclonal MSN1; C, control monoclonal.

TABLE 1. Inhibition of $G_{t\alpha}$ reaction with MSN1 by $G_{t\beta\gamma}$ and rhodopsin

Addition	% Inhibition
(µg)	(mean ± SEM)
$G_{t\beta\gamma}$ (0.03)	11 ± 4
$G_{t\beta\gamma}$ (0.30)	47 ± 7
Rho[L] (0.03)	6 ± 6
Rho[L] (0.30)	80 ± 12
Rho[D] (0.03)	0
Rho[D] (0.30)	0
$G_{t\beta\gamma}$ (0.03) + Rho[L] (0.03)	29 ± 4

$G_{t\alpha}$ (0.1 µg) was mixed with either 0.03 or 0.3 µg of $G_{t\beta\gamma}$, light-activated (Rho[L]) or dark-adapted (Rho[D]) rhodopsin. Samples were applied to nitrocellulose filters and reaction with MSN1 was assessed using a peroxidase-based detection system (12). All proteins were in buffer containing 20 mM Tris (pH 7.5), 0.5 mM $MgCl_2$, 0.05 mM EGTA, 0.5 mM sodium azide, and 1 mM dithiothreitol. Rhodopsin was activated by exposure to room lighting for 10 min. Immunoreactivity was quantified by densitometry and the percentage of inhibition was calculated relative to the reactivity of $G_{t\alpha}$ alone.

tory effect on immunoreactivity is compatible with the conclusions that (i) the MSN1 epitope includes both the amino and carboxy termini, (ii) binding of rhodopsin at the carboxy terminus alters the conformation of the epitope at the amino terminus, or possibly, (iii) the amino and carboxy termini are in close proximity such that binding of rhodopsin to $G_{t\alpha}$ partially blocks the epitope. Inhibition of ADP-ribosylation by MSN1 may result from its binding at either the amino terminus, resulting in the disruption of $G_{t\beta\gamma}$ binding, or at the carboxy terminus where, conceivably, it may interfere with the accessibility of the ADP-ribose acceptor site to PT.

Immunological characterization of the interaction of $G_{t\beta\gamma}$ and $G_{t\alpha}$. Monoclonal antibody 2H3, against the $G_{t\gamma}$ subunit, inhibited rhodopsin-stimulated GTP hydrol-

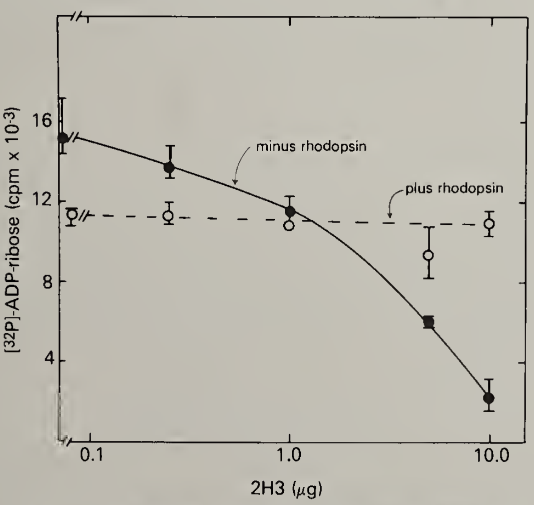

FIG. 5. Effect of antibody 2H3 on rhodopsin-stimulated GTP hydrolysis. Kanaho at al. (17) describe conditions for assay of GTP hydrolysis; the data used are from Halpern and Moss (11).

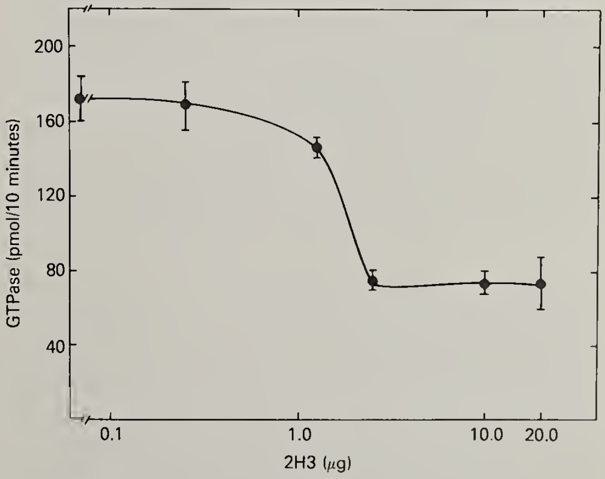

FIG. 6. Effect of antibody 2H3 on PT-catalyzed ADP-ribosylation of transducin. The data used are from Halpern and Moss (11).

TABLE 2. Effect of mutagenesis at the carboxy terminus of $G_{o\alpha}$ on PT-catalyzed ADP-ribosylation

$G_{o\alpha1}$ species	Toxin-catalyzed ADP-ribosylation
WT	+
M:Cys^{351}→Gly	–
M:Leu$^{353}\Delta$, Tyr$^{354}\Delta$	–
M:Tyr$^{354}\Delta$	+
M:Tyr$^{354}\Delta$, Leu^{353}→Gly	–
M:Tyr$^{354}\Delta$, Leu^{353}→Ala	–

Recombinant $G_{o\alpha1}$ mutants were prepared by modifying the methods of Zoller and Smith (41), Taylor et al. (34), and Higuchi et al. (13) and expressed in *E. coli* utilizing the vector pRC23 (6). Cells were grown, lysed, and a DEAE fraction obtained (C. W. Angus, J. Avigan, J. Moss, and M. Vaughan, unpublished data). This material was assayed for ADP ribosylation (39). All samples exhibited equivalent immunoreactivity with $G_{o\alpha}$ antibody. M, Mutant; Δ, Deletion.

ysis (Fig. 5) and PT-catalyzed ADP-ribosylation of $G_{t\alpha}$ in the presence of $G_{t\beta\gamma}$ (Fig. 6). Rhodopsin blocked inhibition of ADP-ribosylation by 2H3 (Fig. 6); this is consistent with the hypothesis that the epitope on $G_{t\gamma}$ for 2H3 is in proximity to the carboxy-terminal rhodopsin-binding site of $G_{t\alpha}$.

Mutagenesis of the $G_{o\alpha1}$ carboxy terminus. Oligonucleotide-directed mutagenesis was performed at the carboxy terminus to yield several mutant $G_{o\alpha1}$ proteins. These mutants were partially purified and their ability to serve as PT substrates in the absence of βγ was evaluated. As noted in Table 2, no ADP-ribosylation was observed when the Cysteine-351 was replaced with Glycine. Deletion of the two carboxy-terminal amino acids also resulted in a loss in ADP-ribosylation, whereas deletion of the terminal Tyrosine was far less significant and resulted in a protein that was ADP-ribosylated. Deletion of the terminal Tyrosine and substitution of an Alanine or Glycine for the now-terminal Leucine had a significant negative effect on the toxin-catalyzed reaction (Table 2).

DISCUSSION

This report addresses (i) the molecular basis for the heterogeneity of $G_{o\alpha}$ mRNA and (ii) the requirements for PT-catalyzed ADP-ribosylation of $G_{o\alpha1}$ and $G_{t\alpha}$. As demonstrated previously (28), hybridization of poly(A)$^+$ RNA with oligonucleotide probes representing the common region of the alternatively spliced forms of $G_{o\alpha}$ resulted in a complex banding pattern unlike that seen thus far with most G_α subunits. Based on recent studies, all of these bands appear to be the product of one $G_{o\alpha}$ gene (Murtagh et al., J. Cell Biol. 107:4015, 709a). All of the bands hybridized with $G_{o\alpha}$-specific oligonucleotide probes complementary to sequences near the center of the coding region and in the 5′-untranslated region. Determination of the molecular basis for the hybridization pattern was facilitated by isolating $G_{o\alpha}$ cDNAs which differed in the carboxy termini of the coding region and in the 3′-untranslated regions (16, 28, 31). By using specific oligonucleotide probes directed to these regions, it was possible to classify the four major bands. The 6.2–kb mRNA species corresponds to the $G_{o\alpha2}$ carboxy-coding domain, whereas the bands at 4, 3, and 2 kb all correspond to $G_{o\alpha1}$. The latter three bands appear to originate from alternative splicing of the $G_{o\alpha1}$ 3′-untranslated regions (28). The 3- and 2-kb mRNA species have relatively unique 3′-untranslated regions, whereas the 4-kb mRNA includes combined segments of the untranslated regions found in the 2- and 3-kb mRNAs (28).

PT-catalyzed ADP-ribosylation of G_α subunits appears to be specific for a Cysteine located four amino acids from the carboxy termini (40). This Cysteine is present in $G_{o\alpha1}$, $G_{o\alpha2}$, $G_{t\alpha1}$, $G_{t\alpha2}$, $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ (27); presumably those G proteins lacking the Cysteine are PT-insensitive. Although it has been reported that free Cysteine is ADP-ribosylated, it apparently is a poor toxin substrate (21). The G_α subunits are not optimal toxin substrates in the absence of βγ which significantly enhances PT-catalyzed ADP-ribosylation of G_α (18, 19, 31, 36) (Fig. 4). βγ is believed to interact, at least in part, at the amino terminus of α. Indeed, proteolytic cleavage (e.g., trypsin) of $G_{i\alpha}$, $G_{o\alpha}$, and $G_{t\alpha}$ resulting in a loss of their amino termini prevented βγ-stimulated ADP-ribosylation (24, 26, 39). Antibodies believed to react in part with the amino terminus of α (e.g., MSN1) inhibited βγ-stimulated ADP-ribosylation (Fig. 4). The MSN1-binding site is significant in that it may involve both the amino and carboxy termini of $G_{t\alpha}$. In support of this hypothesis, it was observed that rhodopsin, which interacts with $G_{t\alpha}$ at the carboxy terminus, inhibited its reaction with MSN1. Moreover, this inhibition by rhodopsin was synergistic with inhibition by $G_{t\beta\gamma}$, which promotes coupling of $G_{t\alpha}$ to receptor. More recent studies on chemical cross-linking of ADP-ribosylated $G_{t\alpha}$ and $G_{t\beta\gamma}$ indicate that the γ subunit is in close proximity to the α carboxy terminus (37). Consistent with these findings, it was observed that antibody 2H3, against $G_{t\gamma}$, inhibited ADP-ribosylation; its effect was blocked by rhodopsin, which binds at the carboxy terminus. The immunological data thus support a structural model in which the α-subunit carboxy terminus is located in proximity to its amino terminus as well as to the γ subunit of the βγ complex.

The site of PT-catalyzed ADP-ribosylation is relatively conserved in all the toxin-sensitive substrates. This domain is involved in receptor interaction, and conservation may reflect the fact that receptors coupling to the G_o, G_i, and G_t families share similar functional domains. Regions of these domains, however, might also be involved in recognition of G_α by pertussis toxin. In the present studies, the effects of single amino acid substi-tutions and deletions at the carboxy terminus were examined. As expected, no ADP-ribosylation was observed when the Cysteine was replaced. Deletion of the two terminal amino acids resulted in loss of the ability of the protein to serve as an ADP-ribose acceptor, whereas the protein was still a PT substrate following deletion of the carboxy-terminal Tyrosine. The penultimate Leucine appeared to be much more critical. In the Tyrosine-deletion mutations, replacement of the now-terminal Leucine with Glycine or Alanine resulted in loss of activity. Thus, amino acid substitutions at the penultimate position have profound effects on activity. Further analysis is necessary to determine whether substitutions at other positions near the acceptor site might alter activity and the interactions of α with β and γ subunits.

ACKNOWLEDGMENTS

We wish to thank Carol Kosh for expert secretarial assistance.

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DISCUSSION

DR. BURNS: You showed that when the cystine that is modified by pertussis toxin is changed to a glycine, you can no longer get ADP ribosylation. Have you tried other amino acids that are more similar in structure to cystine?

DR. TSUCHIYA: You mean at the position of 351?

DR. BURNS: Right.

DR. TSUCHIYA: No, we have never done that.

Elucidation of Linear B-Cell Epitopes in the Pertussis Toxin S3 Subunit by Antipeptide Antibodies

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Virulent *Bordetella pertussis* organisms exhibit a remarkable assembly of virulence factors promoting the development of the human respiratory disease whooping cough. Besides cell-surface-associated factors like pili and filamentous hemagglutinin (32), *B. pertussis* also produces a number of exotoxins like pertussis toxin (PT), adenylate cyclase toxin, tracheal cytotoxin, and dermonecrotic toxin (17, 21, 22, 45, 46). Expression of the majority of the known virulence factors is regulated coordinately (9, 18, 34). Most of the symptoms of whooping cough can be induced in mice by the action of PT alone (27, 29, 30, 36). Thus, PT seems not only to be the most prominent virulence factor of the organism (19) but is also supposedly responsible for the rare but occasionally severe side effects seen upon pertussis toxin vaccination with the current whole-cell pertussis vaccines (23, 40). Multiple efforts are under way to develop new and safe vaccines one component of which should be PT which has been inactivated by genetic engineering or efficient detoxification (1, 3, 24, 25, 28, 31, 35).

Pertussis toxin acts through the ADP ribosylation of the α subunit of certain G proteins to inhibit the regulation of the cellular adenylate cyclase which leads to a nonphysiological increase in the cAMP level (8, 21). The genes coding for PT have been cloned and sequenced (15, 16, 26). The single polypeptide chain of the A protomer (S1 subunit) carries the enzymatic activity, whereas the multi-subunit B oligomer (S2, S3, S4 [two], and S5) mediates the docking of the toxin to cellular receptors. The latter is probably also involved in the translocation of the S1 subunit to the cytosol (4, 6, 10, 20, 39, 41). The B oligomer and its two constituting dimers (S2-S4 and S3-S4), but not single, isolated subunits have been shown to be protective in the mouse model for *B. pertussis* infections (2, 25, 36). The B oligomer is probably also responsible for the mitogenic effects and possible "superantigenicity" discussed for PT (42, 44). To investigate the serological properties of subunits constituting the B oligomer and to provide probes for the elucidation and characterization of functional domains as (for example) the carbohydrate-binding sites harbored by the B oligomer, synthetic peptides corresponding to selected segments of the S2 subunit have been prepared (38). The antipeptide antibodies have been used as probes to map linear immunogenic and antigenic determinants and to identify domains involved in the formation of the carbohydrate-binding pocket (37, 38). In the present study, linear antigenic and immunogenic epitopes of the S3 subunit of PT were identified. Because PT harbors a second, distinct binding site, besides S2, S3 also has been implicated in receptor recognition (37).

Throughout this report, the term "antigenic epitope (determinant)" refers to domains in the native toxin that are recognized by antibodies engendered by synthetic peptides cor-responding to this region. "Immunogenic epitope (determinant)" describes linear segments in the toxin that are recognized by the immune system and thus give rise to antibodies that recognize the corresponding synthetic peptides.

MATERIALS AND METHODS

Pertussis toxin. The PT used in this study was either a gift by the Institut Mérieux, Lyon, France, or purchased from List Biochemicals, Campbell, Calif.

Selection of synthetic peptides. The choice of segments of the S3 amino acid sequence to be prepared as synthetic peptides was biased with regard to hydrophilic ß-turns as indicated by secondary structure predictions according to the algorithms of Chou and Fasman (7),

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Robson and Suzuki (33), Kyte and Doolittle (13), and Hopp and Woods (11). A natural or additional Cysteine residue was placed at the end of the amino acid sequence distal to the predicted ß-turn, thus providing a unique site for coupling of the synthetic peptides to protein carriers in a defined orientation by heterobifunctional cross-linking agents (see below).

Characterization of peptides. Peptides were synthesized with Fmoc technology using 4-(hydroxymethyl)-phenoxymethyl-copol(styrene-1% divinylbenzene) resin as solid support (Novabiochem, Läufelfingen, Switzerland). The coupling of each amino acid was monitored with ninhydrin (12) and, if necessary, repeated until >99% efficiency was achieved. After purification by reverse-phase high-pressure liquid chromatography, the identity of each synthetic peptide was verified by amino acid analysis.

Coupling to carrier proteins. Each peptide was coupled to bovine serum albumin (BSA) using succinimidyl-4-(N-maleimido-methyl)-cyclohexane-1-carboxylate (SMCC) and to thyroglobulin, using m-maleimidobenzoyl N-hydroxysuccinimide ester (MBS) as heterobifunctional cross-linker.

Pertussis toxin and peptide antisera. All antisera were prepared in female white chinchilla bastard rabbits. Peptide-carrier conjugate (250 to 300 µg) and 50 µg of free peptide respectively. 25 µg PT in 100 µl of RAS-adjuvants (PAN Systems, Aidenbach, Federal Republic of Germany) were emulsified and injected subcutaneously at multiple sites. Booster injections were given 4 weeks later. At 7 to 8 days after the booster injections, the rabbits were bled from the ear vein. For preparation of specific antipeptide antisera, rabbits were only immunized with the respective peptide-MBS-thyroglobulin conjugates.

Solid-phase antigen-binding assay. Peptide-protein conjugates (1 mg/ml), PT (1 µg/ml), or free peptides (500 µg/ml) were coated on polystyrene microtiter plates in phosphate-buffered saline (PBS). Free binding sites were blocked with 3% BSA-PBS at 4°C overnight. The plates were incubated with dilutions of antisera in 0.1% BSA-PBS and washed thoroughly with 0.03% Brij-35/PBS. Bound antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG), followed by p-nitrophenyl phosphate as substrate. The enzyme reaction was evaluated in an automated enzyme-linked immunosorbent assay (ELISA) reader (Titertek) at 405 nm. All assays were repeated several times in duplicate.

Western blotting (immunoblotting). To separate PT subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the toxin was denatured by heating for 15 min at 100°C in sample buffer (4 M urea, 2% sodium dodecyl sulfate, 0.6 M 2-mercaptoethanol, 4 mM EDTA, 10% glycerol). The electrophoretic transfer was performed in 25 mM Tris-HCl, pH 7.4, essentially as described by Towbin (43) and Burnette (5). After transfer the nitrocellulose was blocked with 5% BSA-PBS overnight at 4°C. Antiserum subsequently was applied in a 1:200 dilution in 0.1% BSA-PBS. After 90 min at ambient temperature, the nitrocellulose was washed four times with 0.03% Brij 35-PBS and incubated with a 1:5,000 dilution of alkaline phosphatase-conjugated second antibody (Dianova, Hamburg) in 0.1% BSA-PBS. Bound antibody was detected with Nitro Blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate substrate in AP buffer (100 mM Tris, pH 9.5, 1 mM Mg). The reaction was stopped with 20 mM Tris (pH 8.0) containing 5 mM EDTA.

RESULTS

Choice and synthesis of peptides. Fourteen synthetic peptides (R1-12, R12-23, R23-32, R14-29m, R14-29, R36-51, R53-63, R87-95, R95-107, R104-117, R120-130, R134-150, R147-160, and R164-178) have been prepared by the solid-phase technique for the elucidation of linear antigenic and immunogenic determinants in the 199 amino acid sequence of the PT S3 subunit. The complete amino acid sequences of the synthetic peptides compared with their corresponding segments in the highly homologous S2 subunit are depicted in Table 1. The sequences to be synthesized were chosen according to their probability to encompass hydrophilic ß-turns as predicted by different secondary structure prediction algorithms (7, 11, 13, 34) (Fig. 1). All peptides were synthesized with either a natural or an additional Cysteine residue at either end distal to the predicted ß-turn, thereby facilitating coupling in a unique orientation to a carrier protein.

Peptides as antigens. Each of the 14 peptide-BSA conjugates as well as the free peptides were assessed by ELISA with dilutions of polyclonal anti-PT antiserum. Peptides recognized by anti-PT antibodies are defined to encompass immunogenic determinants. Anti-PT antiserum recognized 4 of the 14 peptides (Fig. 2A and B). Sequences R36-51 and R134-150 harbor major linear immunogenic determinants, whereas segments R87-95, R120-130, and R14-29 encompass rather minor immunogenic epitopes (Table 2). Peptides corresponding

TABLE 1. Sequence comparison and location of the S3 synthetic peptide sequences with the corresponding segments of the S2 subunit

PT subunit	Amino acids	Sequence
S3	1-12	VAPGIVIPPKAL-**C** [a]
S2		ST-------QEQ
S3	12-23	LFTQQGGAYGR**C** [b]
S2		QI--H-SP----
S3	23-32	**C**PNGTRALTV
S2		-A-K------
S3	14-29m	TQQGGAYGR**C**PNGTRA
S2		--H-SP----A-K---
S3	14-29	**C**-TQQGGAYGR**C**PNGTRA
S2		--H-SP----A-K---
S3	36-51	**C**-RGNAELQTYLRQITPG
S2		--SGD--E---HV-R-
S3	53-63	**C**-SIYGLYDGTYL
S2		--FA-------
S3	87-95	**C**ITTIYKTG
S2		--M-TRN--
S3	95-107	**C**-GQPAADHYYSKVT
S2		----T-----N--
S3	104-117	SKVTATRLLASTNS-**C**
S2		-N-------S----
S3	120-130	CAVFVRDGQSV
S2		------S--P-
S3	134-150	**C**ASPYEGRYRDMYDALR
S2		-T---D-K-WS--SR--
S3	147-160	DALRRLLYMIYMSG-**C**
S2		SR--KM--L--VA-
S3	164-168	**C**-RVHVSKEEQYYDYED
S2		---------------

[a] - **C** indicates a purposely added Cysteine residue.

[b] **C** indicates the Cysteine residue used for specific coupling of the peptide to carrier proteins.

— denotes an identical amino acid.

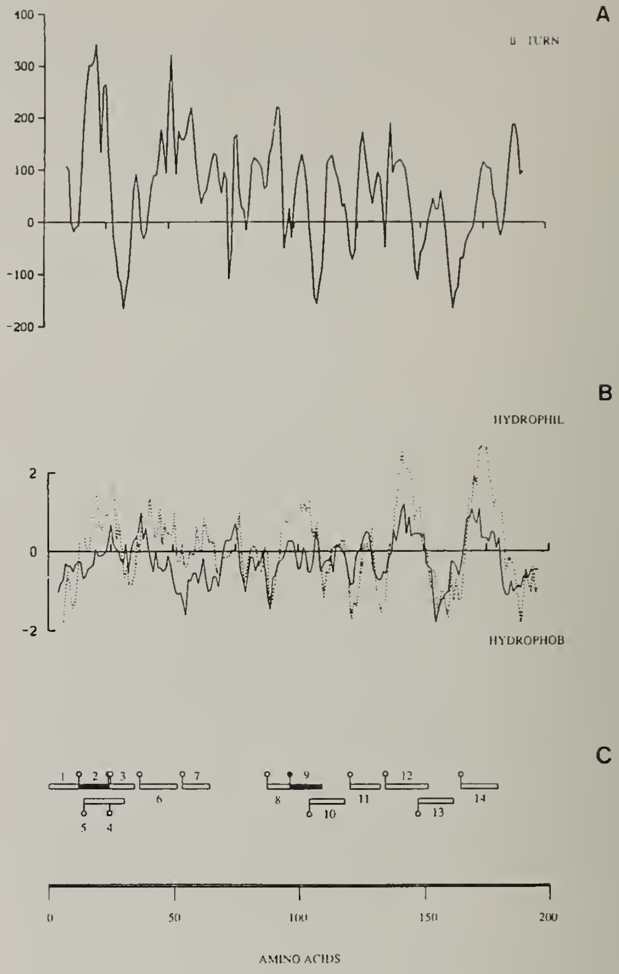

FIG. 1. Predictive analysis of (A) the local average ß-turn potentials and (B) the hydrophilicity according to Hopp and Woods (11) (——) and Kyte and Doolittle (13) (------) of the S3 amino acid sequence. The hydrophilicity values are derived from heptapeptide moving average and are plotted at the midpoint of each section. (C) Synthesized amino acid sequences; (φ) indicates the conjugation site via a natural or additional Cysteine residue. The amino acid numbering is given at the bottom of the figure.

to residues R36-51 and R87-95 show different degrees of recognition depending on whether they are coated onto the microtiter plate as free peptides or as peptide-BSA conjugates (Fig. 2A and B).

Peptides as immunogens. Polyclonal antipeptide antisera were produced in rabbits with peptide-MBS-thyroglobulin conjugates and were assessed for their capacity to recognize the homologous peptide-SMCC-BSA conjugates as well as PT, thus defining antigenic determinants. All peptides elicited a strong antipeptide response (data not shown). Several peptides were found to elicit antibodies cross-reacting with solid-phase bound PT (Fig. 3). Strongly cross-reactive antisera were induced with R1-12, R164-178, R12-23, and R147-160 (Fig. 3A); R14-29m coupled to carrier proteins through an internal Cysteine residue induced a medium response as did R95-107. Interestingly, peptide R14-29 which has been conjugated via an additional N-terminal Cysteine did not engender cross-reactive antibodies. Synthetic peptides corresponding to the N- and C-termini of the S3 subunit are not recognized by the anti-PT antiserum, indicating that these regions in the native protein are not available for the immunosystem and thus are immunorecessive. A rather weak cross-reactive response

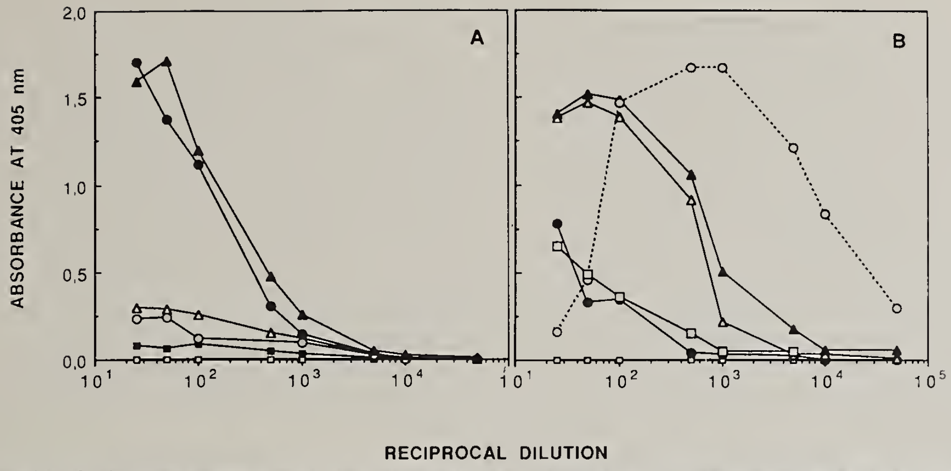

FIG. 2. Reaction of polyclonal anti-PT antiserum with (A) peptide-BSA conjugates and (B) free synthetic peptides by ELISA, as described in text. The A_{405} was corrected (-□-) for nonspecific binding due to pre-immune sera and BSA. Symbols: -■-, R14-29; -●-, R36–51; -△-, R87-95; -○-, R120-130; -▲-, R134-150; -□-, R147-160; ··○··, PT.

TABLE 2. Linear immunogenic and antigenic determinants of the PT S3 subunit

No.	Amino acid	Sequence	Determinant[a]		
			Immunogenic	Antigenic	
				Western	ELISA
1	1-12	VAPGIVIPPKAL-C[b]	–	++	++
2	12-23	LFTQQGGAYGR-C[c]	–	+	+
3	23-32	CPNGTRALTV	–	–	–
4	14-29m	TQQGGAYGRCPNGTRA	–	+	+
5	14-29	C-TQQGGAYGRCPNGTRA	–	–	–
6	36-51	C-RGNAELQTYLRQITPG	+	+	(+)
7	53-63	C-SIYGLYDGTYL	–	–	+
8	87-95	CITTIYKTG	+	–	–
9	95-107	C-GQPAADHYYSKVT	–	+	+
10	104-117	SKVTATRLLASTNS-C	–	–	+
11	120-130	CAVFVRDGQSV	–	–	–
12	134-150	CASPYEGRYRDMYDALR	+	+(+)	+
13	147-160	DALRRLLYMIYMSG-C	+	–	+
14	164-178	C-RVHVSKEEQYYDYED-	–	++	++

[a] Immunogenic epitopes denote segments in the native toxin that induce antibodies capable of recognizing the corresponding synthetic peptides. Antigenic epitopes refer to segments of the protein that, as synthetic peptides, give rise to antibodies reacting with the holotoxin.

[b] -C indicates a purposely added Cysteine residue.

[c] C indicates the Cysteine residue used for specific coupling of the peptide to carrier proteins.

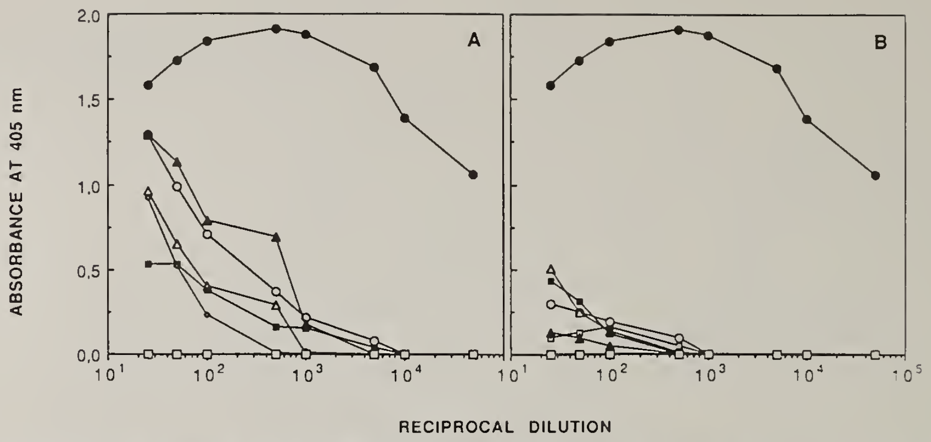

FIG. 3. Recognition of intact PT by antiserum against peptide-MBS-thyroglobulin conjugates and PT. The A_{405} has been corrected for unspecific binding as in Fig. 2; -●- indicates binding of antitoxin antiserum to PT. (A) antipeptide sera exhibit significant binding. Symbols: -O-, R1-12; -△-, R12-23; -■-, R14-29m; -◇-, R147-160; -▲-, R164-178. (B) peptide antisera with rather weak binding to PT. Symbols: -⊡-, R36-51; -O-, R53-63; -△-, R95-107; -▲-, R104-117; -■- R134-150.

was found when peptides R134-150, R53-63, R36-51, and R104-117 were used as immunogens and the antipeptide antisera were assayed in ELISA (Fig. 3B). Antibodies engendered against peptides R23-32, R14-29, R87-95, and R120-130 did not recognize the holotoxin in either ELISA or by Western blotting.

Recognition of antigenic determinants by Western blotting. To investigate whether the antipeptide antisera also recognized the S3 subunit under-denaturing conditions, PT was boiled in urea-sodium dodecyl sulfate-2-mercaptoethanol by the method of Laemmli (14) and Tamura et al. (41), electrophoresed in 15% sodium dodecyl sulfate-polyacrylamide gels, and transferred to nitrocellulose (Fig. 4). Antisera engendered against peptides R1-12, R36-51, R134-150, and R164-178 showed a strong reaction with S3, indicating that these antigenic determinants were not destroyed under harsh denaturing conditions. Antisera directed against peptides R12-23,

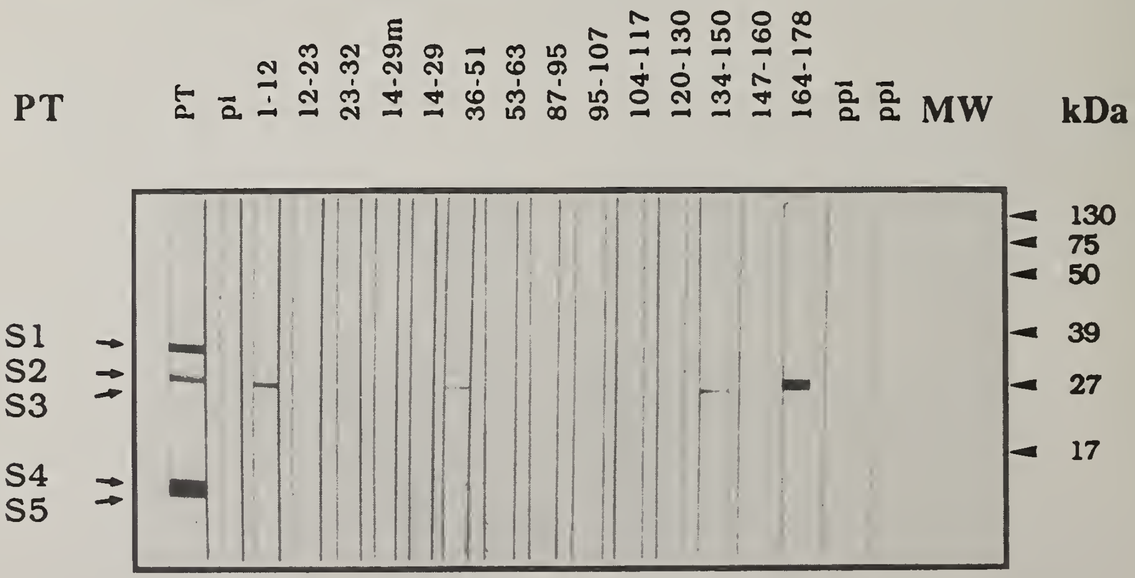

FIG. 4. Western blot of PT with antipeptide antisera diluted 1:200 in 0.1% BSA-PBS. Alkaline phosphatase-conjugated goat anti-rabbit antibodies and Nitro Blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate were used to detect bound antibodies.

R14-29m, and R95-107 showed only a weak cross-reactivity with S3 under denaturing conditions.

Recognition of S2 by anti-S3 peptide antibodies. Despite the remarkable degree of sequence homology between S3 and S2 (70%) which is reflected in the sequences chosen for duplication as synthetic peptides (Table 1), only antibodies raised against the synthetic peptide R164-178, which is identical in both subunits, also recognized S2 in Western blotting (Fig. 4). All other antipeptide antisera were specific for the S3 subunit, further indicating that, even under relatively strong denaturing conditions, the structural heterogeneity of the two homologous subunits is preserved.

DISCUSSION

Among the striking number of known virulence factors exhibited by *Bordetella pertussis* organisms, the exotoxin PT is regarded as one of the major factors involved in the development of the pathology of whooping cough, acting not only as an exotoxin but also as a hemagglutinin (21, 22, 39). Furthermore, contaminating active toxin has been held responsible for some rare complications associated with the current whole-cell pertussis vaccines. Although trials conducted with an acellular component vaccine consisting of filamentous hemagglutinin and PT have shown the component vaccine to be efficient, they have failed to settle the issue of possible side effects intrinsic to preparations from biological sources (28). The cloning and sequencing of the genes coding for PT expression has opened the possibility of using synthetic peptides for the identification and characterization of immunogenic and antigenic determinants harbored by the single subunits of PT. The identification of segments carrying antigenic epitopes will be helpful for the development of a fully synthetic vaccine against whooping cough based in part on PT.

Though most determinants in proteins are conformational and more often represented by discontinuous rather than continuous stretches of amino acids, synthetic peptides can be successfully applied as structure function probes if they elicit antibodies that recognize the native or denatured protein with sufficiently high affinity. In this approach, the synthetic peptides should adopt, or should be induced to adopt, conformations that are identical (or at least very similar) to that of the corresponding segment in the parent protein. This implies that the linear immunogenic and antigenic determinants detected with antipeptide antibodies certainly do not represent the complete repertoire of determinants harbored in a given protein.

The present study describes the use of 14 synthetic peptides corresponding to linear sequences predicted to represent hydrophilic ß-turns of the PT S3 subunit; this subunit has been proposed to harbor a second receptor binding site parallel to S2 (37). Four linear immunogenic and five linear antigenic determinants have been identified using antipeptide antisera as reagents. The locations of these immunogenic and antigenic determinants are summarized in Table 2. Major linear immunogenic determinants have been identified in peptides R36-51, R87-95, and R134-150; a minor immunogenic epitope was detected in peptide R147-160. Interestingly, recognizing two of these synthetic peptides by antitoxin antiserum changes drastically depending on the coating of the ELISA plates either with the peptide-BSA conjugates or with free peptides. Peptide R87-95 is only weakly recognized when coated as BSA-conjugate but shows strong binding of antitoxin antiserum when applied as free synthetic peptide. The opposite is true for peptide R36-51, which is strongly recognized as BSA-conjugate but reacts rather weakly as free peptide. These differences in reactivity might be attributed to different recognition sites available on the peptides for antibody binding which are not occupied due to hydrophobic interactions with the polystyrene support. Thus, in evaluating ELISA results, both types of assays should be taken into account.

Only peptides R36-51 and R134-150 correspond to linear segments in the S3 subunit that harbor both immunogenic and antigenic determinants. Particularly, peptides corresponding to the segments at the N-terminus and near the C-terminus of the S3 subunit are not recognized by polyclonal antitoxin antiserum. Thus, these segments are denoted as "immunorecessive" in the native PT; as synthetic peptides, however, they elicit antisera that show strong binding to PT in Western blotting and ELISA. Antigenic determinants have been detected in peptides R1-12, R12-23, R14-29m, R36-51, R95-107, R134-150, and R164-178, again demonstrating that antibody populations can be induced with synthetic peptides which are not available by immunization with the native protein.

As has been found with synthetic peptides corresponding to segments of the S2 subunit, only one of the seven antipeptide antisera reactive with the S3 subunit in Western blotting also binds to the S2 subunit (R164-178). This segment has an identical amino acid sequence in the S2 and S3 subunits (Table 1). All other synthetic peptides show alterations between the S3 and S2 amino acid sequences which might interfere with recognition by heterologous antipeptide antibodies. The two PT subunits might also retain structural heterogeneity even under the strong denaturing conditions experienced during Western blotting.

It will be interesting to see whether the antipeptide antibodies elicited by peptides corresponding to antigenic determinants will be able to interfere with toxin binding reactions and will thus serve as structure-function probes for mapping the second receptor recognition site harbored by the S3 subunit of PT.

ACKNOWLEDGMENTS

This work was supported by grant BCT 0381-5 of the Bundesminsterium für Forschung und Technology, Federal Republic of Germany, and by a grant from the Institut Mérieux, Lyon, France.

We are indebted to Elke Bernarth and Rüdiger Pipkorn of the Zentrum für Molekulare Biologie, Universität, Heidelberg, for peptide synthesis and to Ute Seitz for technical assistance.

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DISCUSSION

DR. BURNS: You, and I think a lot of people suggest that there are two sites on the pertussis toxin molecule that bind, one site on S3-S4 and one site on S2-S4. Do you have any feeling for how rather different cell types interact preferentially with the two different sites or could you give us your thoughts on that?

DR. SCHMIDT: I must say we did not check any different cell types as maybe you did. I mean, our bias or our idea that it is two different binding sites are more or less the outcome of these peptide studies because, as I said, with the S2 peptides, we could block any binding specificity which is depending on the S2 subunit or S2-S4 dimer alone but we have still a residue of binding or specific binding activity which I think is due to a binding pocket in the S3 or S3-S4 dimer.

DR. BURNS: The other thing is that S2-S4 or S3-S4 will hemagglutinate cells. How do you envision that happening? Do you see more than one binding site on each of the dimers?

DR. SCHMIDT: I would not say. My hypothesis is that in each of these dimers has one binding site. But as you see with the B-oligomer or isolated S5 subunits, those S5 subunits are very sticky molecules, I would rather prefer to think that hemagglutination is due to one isolated dimer is due to dimerization.

Purification, Characterization, and Immunological Evaluation of the 69-kDa Outer Membrane Protein of *Bordetella pertussis*

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Acellular pertussis vaccines have been introduced and used extensively in Japan since 1981. Two types of vaccines are available today. The B type (pertussis toxin [PT]-rich) contains about 25 µg of inactivated PT (as protein) and 25 µg of filamentous hemagglutinin (FHA). The T type (FHA-rich) contains about 3 µg of inactivated PT, 35 µg of FHA, and other bacterial components which are co-purified with these antigens. Analysis of the additional bacterial components present in the vaccine manufactured by Takeda Chemical Industries, Ltd., Hikari, Japan, showed the presence of traces of agglutinogens and significant amounts of the 69-kDa outer membrane protein (OMP). The identification of the 69-kDa OMP from *Bordetella pertussis* (4) and research on the possible function of this protein was initiated by the discovery of à related 68-kDa OMP in *B. bronchiseptica* (14), which confers protective immunity to susceptible animals (16). Therefore, the possible contribution of the 69-kDa OMP to the protective efficacy of acellular vaccines has been suggested, which has triggered further studies on this potentially useful vaccine component (17, 19).

This report describes the extraction and purification of the 69-kDa OMP from fermentor-grown *B. pertussis* and describes biochemical and structural studies of the purified protein. It also evaluates the experimental and immunological properties relevant to the use of the 69-kDa OMP as a pertussis vaccine component in addition to PT and FHA.

MATERIALS AND METHODS

Reagents. Chemicals and solvents used in this study were either analytical grade or high-performance liquid chroma-tography (HPLC) grade. All electrophoresis reagents were obtained from Bio-Rad Laboratories, Richmond, Calif. L-1-Tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin and carboxypeptidase Y were obtained from Sigma Chemical Co., St. Louis, Mo.; and Boehringer Mannheim Biochemicals, Indianapolis, Ind., respectively. Membranes of polyvinylidene difluoride (Immobilon) were purchased from Millipore Corp., Bedford, Mass. Reagents and solvents used for liquid-phase sequencing and for phenylthiohydantoin (PTH)-amino acid identification were obtained from Applied Biosystems Inc.

69-kDa preparation. The 69-kDa protein was obtained by growth of *B. pertussis*, Tohama phase I strain, in modified Stainer-Scholte medium supplemented with heptakis-(2, 6-0-dimethyl)-β-cyclodextrin (10, 11). After batch adsorption of PT and FHA, the cells were collected and washed. The protein was then released by heat treatment, and the extract was clarified by centrifugation of the inactivated cells. The OMP was purified using conventional chromatographic sorbents following a modification of the procedure of Gould-Kostka et al. (8). Affinity chromatography based on monoclonal or polyclonal antibodies against 69-kDa protein was not used. The recovery of purified material was about 50% with yields of 5 to 10 mg of purified product per liter of culture. The 69-kDa and FHA preparations used for immunization and challenge studies were treated with formalin, to inactivate traces of pertussis toxin, if present. The purity of the antigen was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13), followed by Coomassie Blue R-250 staining, Western blot, and isoelectric focusing (IEF) analysis. Specific assays were conducted to detect trace amounts of adenylate cyclase (12), PT (9), and heat-labile toxin (15). Endotoxins were assayed using the *Limulus* amoebocyte test (Bioproducts, Whittaker, Mass.).

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Limited tryptic digestion. Digestion of 500 µg of the 69-kDa protein with L-1-Tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin was performed in 0.050 M ammonium bicarbonate (pH 8.2) for 15 min at room temperature with an enzyme:substrate ratio of 1:1,000. The digested material was then lyophilized.

C-terminal sequence analysis. Digestions of the 69-kDa protein with carboxypeptidase Y were performed in 0.1 M pyridine acetate buffer (pH 5.6) containing 1% SDS at an enzyme: substrate ratio of 1:100. Aliquots, containing 10 nmol of sample, were withdrawn at appropriate time intervals, and the digestion was terminated by the addition of acetic acid. The aliquots were lyophilized and redissolved in 0.2 M of sodium citrate buffer (pH 2.2) and applied directly to the amino acid analyzer (LKB 4151 Alpha Plus).

Two-dimensional gel electrophoresis. Two-dimensional PAGE was carried out as described by Bravo (3) and Bauw et al. (2). The nonequilibrium pH gel electrophoresis was done in tubes with a 1.5-mm inner diameter. Gels containing SDS, which served as the separation medium for the second dimension, were cast in 2-mm-thick slab gels. The separation gel was 12.5% acrylamide to 0.07% bisacrylamide to 0.375 M Tris-HC1, pH 8.7, to 0.1% SDS. The stacking gel was 5% acrylamide to 0.13% bisacrylamide to 0.125 M Tris-HCl, pH 6.8, to 0.1% SDS. The electrode buffer was 0.05 M Tris to 0.19 M glycine to 0.1% SDS, pH 8.3.

Protein electroblotting. Protein electroblotting on polyvinylidene difluoride membranes was carried out as described by Bauw et al. (2). Protein electroblotting on polybrene-coated glass-fiber sheets was performed as described by Vandekerckhove et al. (20). The electrotransfer was done with a transfer buffer of 50 M Tris/50 M boric acid (pH 8.3) using a Bio-Rad Trans-Blot apparatus set at 40 V for at least 8 h. Immobilized proteins were visualized by amido black or Coomassie Blue staining (20). Protein spots were cut out and stored at -20°C.

N-terminal sequence analysis. The 69-kDa protein and limited tryptic peptides were dissolved in 40 µl of a 12% aqueous solution of trifluoroacetic acid before loading on a precycled polybrene-coated glass filter. The sequence analysis was carried out with a pulse-liquid phase-sequencer (model 477A, Applied Biosystems) equipped with an on-line PTH-amino acid derivative analyzer (model 120A) and run with the sequencing program recommended by the manufacturer. Protein spots from the two-dimensional blot (± 3 x 3 mm pieces) were placed into the chamber of the upper cartridge block of the sequencer. No polybrene was added, and the sequencer was operated using the regular sequencing program.

Electrospray mass spectrometry (ESMS). Electrospray mass spectra were recorded on a Sciex API-III triple quadrupole mass spectrometer fitted with a standard pneumatically assisted nebulization probe and an atmospheric pressure ionization source (Sciex, Ontario, Canada). The samples were dissolved at ca. 20 pmole/µl in 30% methanol, 70% H_20, 2% acetic acid (vol/vol/vol) and introduced into the mass spectrometer by infusion at 2 to 3 µl/min. Approximately 4 to 11 min of data were recorded and ca. 150 to 675 pmole of the sample consumed.

Western blot analysis. The 69-kDa protein preparation was analyzed by immunoblot using monoclonal antibody (Mab) BPE3 (4). Mab BPE3 was obtained from the Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md.

Immunogenicity in mice. Groups of 10 Swiss OF1 mice were injected intraperitoneally with dilutions of the adsorbed antigen. Sera were obtained after 28 days and assayed for anti-69 kDa by a sandwich enzyme-linked immunosorbent assay test. Titers were the highest dilution giving an optical density of ≥ 0.5; the ED_{50} was calculated by recording the number of responders at a serum dilution of 20-fold.

Intranasal challenge. Groups of 10 5-week-old OF1 mice were injected intraperitoneally on days 0 and 21 with different vaccine formulations adsorbed to aluminum hydroxide. One week after the booster dose, the mice were challenged by intranasal instillation of about 10^5 to 10^6 CFU of *B. pertussis* strain 18323. Two weeks later, lungs were removed aseptically and individually homogenized in 2 ml of a 1% Casamino Acid solution. Next, 100 µl of serial tenfold dilutions of the lung homogenates were plated onto Bordet-Gengou medium. After 72 to 96 h of incubation at 37°, the number of CFUs was determined. Control mice were injected with the aluminum hydroxide suspension.

Protection was assessed in three different ways: (i) by recording the number of noninfected mice (<10 CFU/lung), (ii) by recording the decrease of the mean CFU in the lungs, or (iii) by giving each mouse a score (5, 4, 3, 2, and 1) corresponding, respectively, to <10, 10

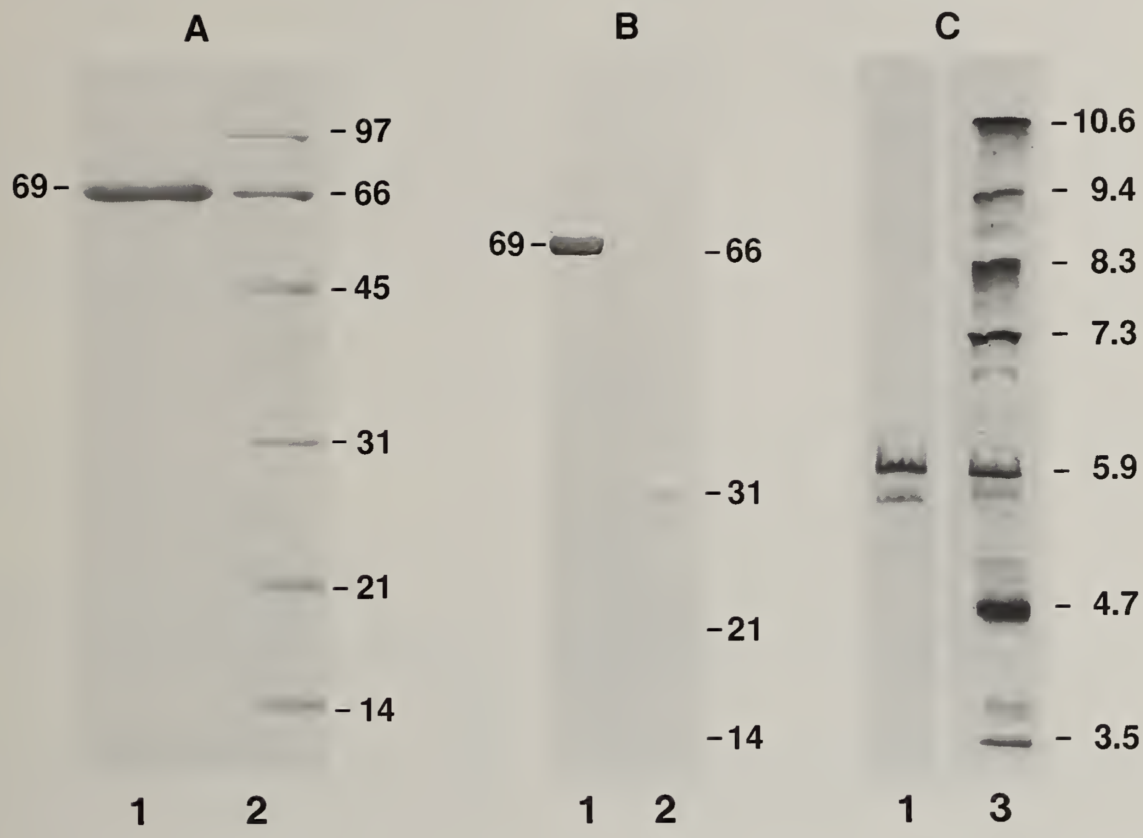

FIG. 1. Identity and purity of the *B. pertussis* 69-kDa protein. (A) SDS-polyacrylamide gel stained with Coomassie Blue. (B) Immunoblot detected with Mab BPE3. (C) Isoelectric focusing gel. Lane 1, purified 69-kDa protein; lane 2, molecular weight markers (in kDa); lane 3, pI references. The position of the 69-kDa band is indicated.

- 10^2, 10^2 - 10^3, 10^3 - 10^4, and >10^4 CFU per lung. The protection rate was defined as the ratio of the sum of individual scores to the maximum theoretical score (score 5 x nb mice). The lethal intranasal challenge tests were performed by N. Guiso at the Institut Pasteur (Paris) as described by Alonso et al. (1).

Intracerebral challenge. The mouse intracerebral challenge test was performed as described in the European Pharmacopoeia. Groups of 12 5-week-old OF1 mice were immunized intraperitoneally with 0.5 ml of serial fivefold dilution (in phosphate-buffered saline) of different vaccine formulations adsorbed to aluminum hydroxide. Two weeks later, mice were challenged intracerebrally with 8×10^4 CFU of *B. pertussis* strain 18323 (5,000 LD_{50}) contained in 30 µl of a 1% Casamino Acid suspension. Mortality was recorded during the next 2 weeks. Mice that died within 48 h after challenge were excluded. A monovalent whole-cell pertussis vaccine (Behringwerke, Marburg, Germany) containing 1 IU/dose was used as reference.

RESULTS

Purity of the 69-kDa antigen. Analysis by SDS-PAGE and Western blot of the purified 69-kDa protein showed one major band with an apparent molecular weight close to 69 kDa (Fig. 1A and B). By IEF in polyacrylamide gel, purified preparations showed two major regularly spaced bands at pI values of 6.0 and 5.7 and occasionally a faint band at 6.3 having all the same apparent molecular size (Fig. 1C). The antigen was deemed 99% pure by size-exclusion HPLC.

Pertussis toxin activity in purified 69-kDa OMP was below the detection limit of the Chinese hamster ovary cell-clustering assay which corresponds to less than 3 ng PT/mg protein. The antigenic preparation was determined to be free of heat-labile toxin and adenylate cy-

TABLE 1. N-terminal sequence studies of the purified 69-kDa protein and its two isoelectric forms

Step	PTH-amino acid	Purified 69-kDa yield (pmol)	pI 6.0 form yield (pmol)	pI 5.7 form yield (pmol)
1	D	650	12	41
2	W	136	3	4
3	N	81	8	23
4	N	128	13	32
5	Q	118	7	20
6	S	108	4	11
7	I	92	7	17
8	V	128	12	25
9	K	88	2	6
10	T	128	5	14
11	G	147		
12	E	55		
13	R	48		
14	Q	70		
15	H	13		
16	G	136		
17	I	55		
18	H	13		
19	I	58		
20	Q	44		

The purified 69-kDa species and the immobilized pI 6.0 and pI 5.7 forms obtained by IEF were submitted to automated Edman degradation (20 and 10 cycles, respectively). Cycle yields are indicated in pmoles of PTH-amino acid.

clase activities. The endotoxin content was below 0.10 endotoxin units per microgram of protein as determined by Limulus amoebocyte lysate assay.

Protein-chemical studies. (i) N-terminal analysis. The purified *B. pertussis* 69-kDa protein was subjected to automated Edman degradation. The identity and yield of the PTH derivatives obtained from 20 cycles are shown in Table 1. The N-terminal sequence corresponds to that predicted from the DNA coding sequence (5).

The two major forms of the 69-kDa protein with estimated pI of 6.0 and 5.7 were separated by high-resolution two-dimensional electrophoresis. The N-terminal amino acid sequence was obtained by direct electroblotting of the separated proteins onto a solvent-resistant support, followed by excision of the protein spots and sequencing. Both forms revealed the expected N-terminal sequence (Table 1).

(ii) C-terminal analysis. Carboxypeptidase Y digestion was carried out to identify the C-terminal end of the 69-kDa protein. The rate of release of amino acids during the digestion is shown in Fig. 2. Under the conditions used for amino acid analysis, Ser and Asn co-elute and contribute differently to the optical density of the peak. Therefore, the information obtained for these two residues cannot be unambiguously assigned to a particular composition. Assuming, however, that Tyr, Glu, and Trp were released in equivalent amounts, approximately two equivalents of Ala and Leu and two to three equivalents of Ser + Asn were found. Thr was only slowly. These data were compared with the predictable C-terminal sequences derived from the gene sequence of the P.93 precursor (5). This composition was not compatible with any sequence near the putative cleavage site at residue 677 which corresponds to an actual molecular weight of 69 kDa. Instead, compatible sequences were found between residues 587 to 597 and 590 to 600 (Fig. 3). Due to the inherent inaccuracies in carboxypeptidase Y digest analysis and the lack of resolution between Asn and Ser, the data are insufficient to locate the C-terminal position more precisely.

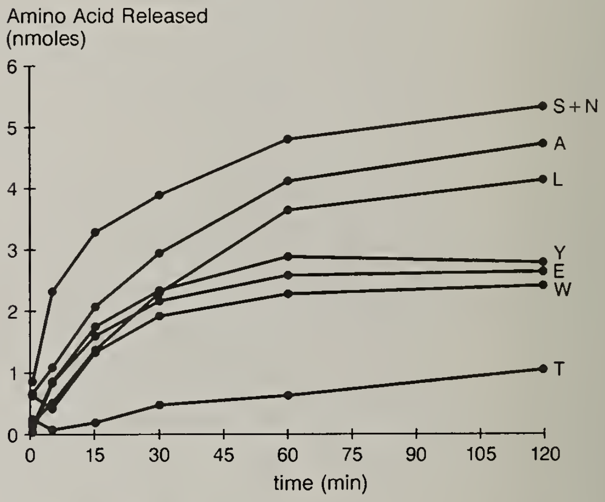

FIG. 2. Carboxypeptidase y digestion of the 69-kDa protein. Rate of release of amino acids from the C-terminal end.

(iii) N-terminal sequence of the 67-kDa product. Preparations of purified *B. pertussis* 69-kDa protein occasionally present a doublet on SDS-PAGE, with the

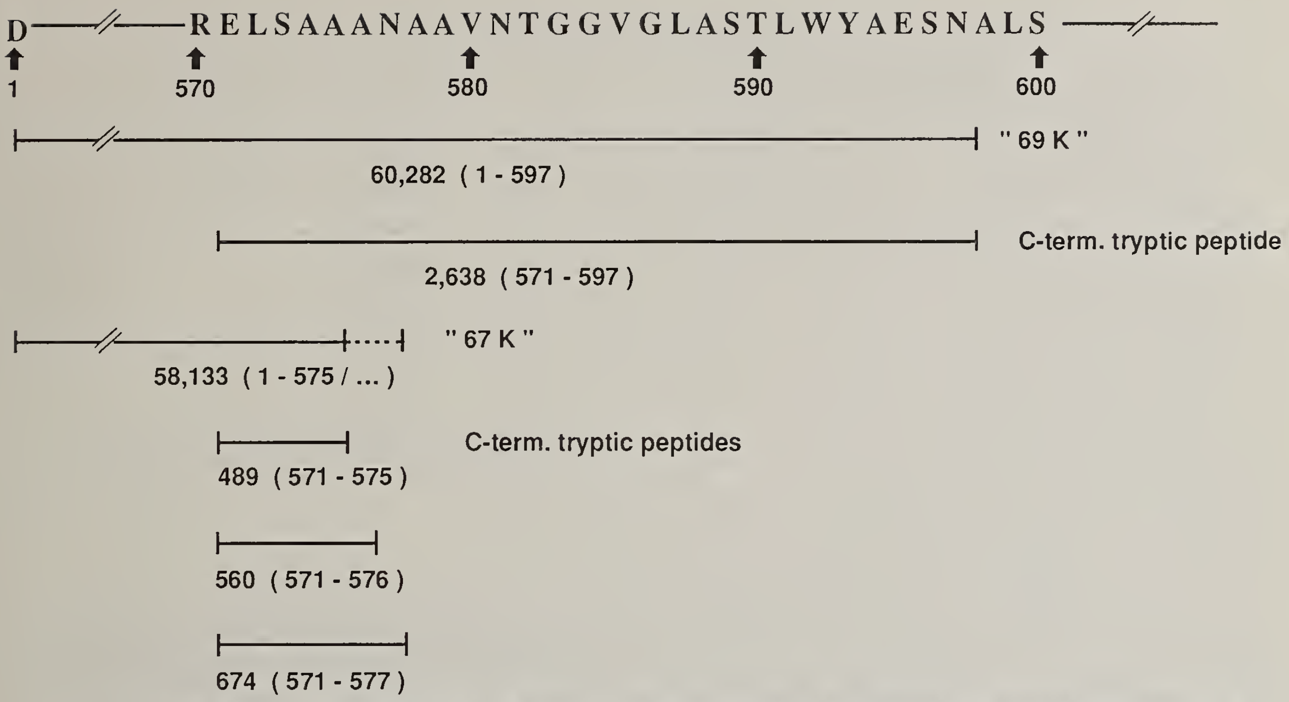

FIG. 3. Sequence studies of the 69-kDa OMP and the related 67-kDa species. Location of the C-terminal tryptic peptides and prediction of the molecular weight of the different fragments identified by sequence determination and ESMS.

upper band corresponding to the 69-kDa species and a lower band at an apparent molecular weight of 67 kDa. Eventually, a complete conversion to the 67-kDa form results. As this phenomenon appeared time dependent, it was assumed that the 69-kDa species is prone to proteolytic cleavage, and the nature of this modification was investigated.

The loss of an approximately 2,000-Da fragment did not modify the reactivity with monoclonal (4) or polyclonal reagents in Western blots. After separation of the 67-kDa species from the 69-kDa band by SDS-PAGE and electrotransfer to polybrene-coated glass-fiber sheets, the N-terminal sequence of the 67-kDa species was determined and found to be identical to that of the complete 69-kDa molecule, implying a C-terminal cleavage in the conversion of the 69-kDa to 67-kDa species.

(iv) Analysis of limited tryptic hydrolyzates. The limited trypsin hydrolysis of the purified 69-kDa protein gave a product with an apparent molecular weight of 67,000 (Fig. 4) which was indistinguishable from the naturally occurring 67-kDa. The mixture of fragments was resolved by size-exclusion HPLC on a Superose 6 column, which gave first a large peak and a small retarded peak (Fig. 5). Sequencing analysis of the first fraction gave the sequence DWNNQSIVKT, which corresponds to the N-terminal sequence of the authentic 69-kDa species. The retarded material gave a readable sequence of 27 amino acids:

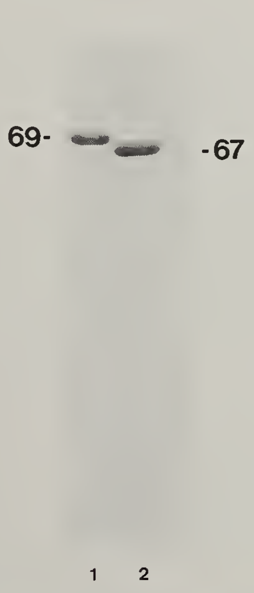

FIG. 4. SDS-PAGE analysis of the limited tryptic digest of 69-kDa protein. Lane 1, purified 69-kDa protein; lane 2, limited tryptic digest. The position of the 69-kDa protein is indicated.

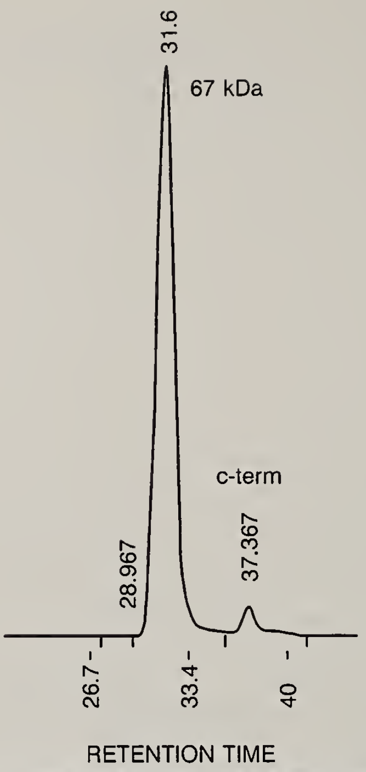

FIG. 5. Size-exclusion HPLC of the limited tryptic digest of 69-kDa protein. The tryptic digest of 69-kDa protein was loaded on a Superose 6HR 10/30 column equilibrated in 50 mM of ammonium bicarbonate (pH 8.2) and eluted at a flow rate of 1 ml/min with the same buffer. Fractions of 1 ml were collected.

ELSAAANAAVNTGGVGLASTLWYAESN. Cycles 28 to 31 failed to deliver interpretable results, with no signal showing the expected yield of PTH-amino acid. This sequence overlaps the C-terminal peptides identified by carboxypeptidase Y, and, unless a premature washout of the peptide under investigation was responsible for the disappearance of signals at cycle 28 and beyond, these results indicate that the C-terminal residue of the 69-kDa protein is at position 597.

A similar experiment was performed on the naturally occurring 67-kDa product in an attempt to localize the natural cleavage site. Trypsin cleavage followed by HPLC also gave a retarded fraction, which generated a readable N-terminal sequence of seven residues: ELSAAAN. The following Edman cycles failed to yield PTH-amino acids with the expected recovery, indicating that the natural cleavage which generates the 67-kDa product occurs beyond the trypsin site at Arg 570, most probably at Asn 577.

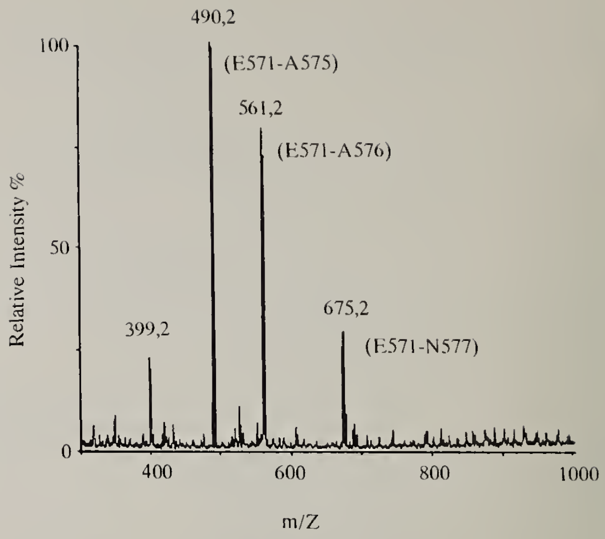

FIG. 6. ESMS analysis of the 67-kDa species C-terminal tryptic peptides. The diagram shows the three signals identifed, their molecular weight, and the corresponding assignment.

Mass spectrometry studies. To confirm the identification of the C-terminal residue of the 69-kDa and 67-kDa forms, the purified preparations were submitted to ESMS. Electrospray produces a coherent series of multiply-charged molecular ions for each protein species present in the sample. If the mass of the charge-carrying species is known (e.g., H = 1.0079), then the number of charges and the mass may be determined simply (6, 7, 18). Furthermore, each peak in a coherent series provides an independent measurement of the mass. This approach extends the useful analytical range to intact proteins above 100 kDa.

The predicted molecular weights of the polypeptides extending to residues 597 to 600, which were identified by carboxypeptidase Y digestion, are 60,282 (597), 60,353 (598), 60,466 (599), and 60,553 (600). These values incorporate the correction of two sequencing errors identified in the amino acid assignment of Charles et al. (5), namely Leu 230 → Pro and Pro 298 → Ser. These differences were detected by amino acid sequence analysis of internal tryptic peptides (results not shown) and correspond to single base pairs replacements.

The intact 69-kDa protein gave signals corresponding to the molecular sizes of 60,297 ± 16 and 60,291 ± 8 in two separate experiments using different protein preparations (Fig. 3). These values match the predicted weight of the polypeptide extending from position 1 to Asn 597. They exclude the alternative locations of the C-terminal

TABLE 2. Immunogenicity in mice

69-kDa OMP[a]			69-kDa OMP + PT, FHA, DT, TT[b]				
				Lot #1		Lot #2	
Dose (µg)	No. of responders (n = 10)	GMT EU/ml	Dose (µg)	No. of responders (n = 10)	GMT EU/ml	No. of responders (n = 10)	GMT EU/ml
20.00	10	2,788					
6.66	8	785	8.000	9	1,215	9	2,537
2.22	10	1,469	4.000	10	1,520	7	1,359
0.74	5	119	2.000	8	814	9	466
0.25	2	<100	1.000	7	382	6	250
			0.500	5	357	7	315
			0.250	0	<100	1	<100
			0.125	0	<100	1	<100
	ED50 = 751 ng			ED50 = 718 ng		ED50 = 541 ng	

[a] Groups of 10 mice received one injection of serial dilutions of the 69-kDa component, formalin-stabilized and adsorbed to aluminium hydroxide.

[b] Eight µg of the 69-kDa component were combined with 8 µg of inactivated PT, 25 µg of FHA, 17 Lf of diphtheria toxoid (DT), and 10 Lf of tetanus toxoid (TT). Serial dilutions of two preparations were given to groups of 10 mice. After determination of antibody titers by enzyme-linked immunosorbent assay, the ED$_{50}$ was calculated by Probit analysis. The number of responders was calculated by reference to an unimmunized control group.

residue, since all the other possible molecular sizes are well beyond the precision limits of these determinations.

To analyze the C-terminal region more precisely, the molecular weight of the C-terminal peptide obtained by limited trypsin digestion was also analyzed by ESMS. These experiments gave the values 2,636 and 2,637 in separate determinations on different preparations. These values closely correspond to the predicted tryptic peptide Glu 571 to Asn 597, which has a theoretical mass of 2,638 and is therefore in agreement with the ESMS result for the intact protein.

Analysis by ESMS of a mixture containing predominantly the 67-kDa species gave an observed mass of 58,132 ± 8, which is consistent with the sequence Asp 1 to Ala 575. The analysis of these tryptic peptides by ESMS gave three signals with observed masses at 489, 560, and 674 (Fig. 6). These signals are consistent with the presence of three different peptides, extending from Glu 571 to Ala 575, Ala 576, and Asn 577, which have the calculated masses of 489.2, 560.3, and 674.3, respectively. These results therefore indicate a ragged C-terminus of the 67-kDa species (Fig. 3).

Evaluation of the 69-kDa OMP as vaccine component. (i) Safety studies. Although it has been established that the purified 69-kDa protein is free of other *B. pertussis* toxins, the general safety of this protein was investigated, since very little is known about its possible activities or metabolic involvements. The safety of the purified 69-kDa protein was therefore studied in groups of 10 BALB/c mice weighing 14 to 15 g, which received three successive subcutaneous doses of 12.5 µg of native or formalin-treated protein adsorbed to aluminium hydroxide. Control groups receiving only saline or the adsorbent were also included. The weights were recorded over a 21-day period and showed no significant differences among groups: the group receiving saline had an average gain of 5.5 g; those receiving aluminium hydroxide increased by 4.8 g; those receiving native or formalin-treated 69-kDa OMP showed an increase of 5.0 or 4.6 g, respectively.

A similar test was performed in 3-day-old OF1 mice receiving one intradermal injection of 10 µg of unadsorbed native or formalin-treated 69-kDa OMP or saline. The groups were randomized to eliminate bias due to suckling. Again, no significant differences in body weight gain at 4 and 7 days after injection were recorded in any of the groups.

(ii) Immunogenicity in mice. The 69-kDa vaccine component was adsorbed to aluminum hydroxide alone (one preparation) or in combination with diphtheria,

Table 3. Mouse intranasal challenge

	Antigens	Dose (µg)	Infection rate	Mean CFU/mouse	Protection rate (%)
A	—	—	10/10	7.1 10^5	4
	Whole-cell vaccine	1/6 HD	1/8	<10	98
	PT	25	7/10	7.7 10^3	62
	PT + FHA	25 + 25	1/10	<10	96
	69-kDa	25	2/9	1.2 10^2	89
B	—	—	7/7	1.3 10^6	3
	Whole-cell vaccine	1/6 HD	1/10	27	96
	69-kDa	1.3	3/10	8.4 10^3	78
	69-kDa + PT	1.3 + 1.3	5/10	1.2 10^3	76
	PT + FHA	1.3 4	1/10	2	94
	69-kDa + PT + FHA	1.3 + 1.3 + 4	1/10	1.3 10^2	94
	69-kDa + PT + FHA	1.3 + 1.3 + 4	0/10	<10	100

Groups of 10 mice received two injections of the indicated combination of antigens adsorbed to aluminum hydroxide at 3-week intervals. One week after the second injection, they were challenged intranasally with the virulent *B. pertussis* strain 18323. Two weeks after challenge, the lungs were aseptically removed and homogenized and dilutions of homogenates plated onto Bordet-Gengou agar medium. After incubation, the number of CFUs per lung and the relative protection score were calculated.

HD, Human dose

tetanus toxoids and the pertussis components inactivated PT and FHA (two preparations). Serial dilutions of these adsorbed antigens were assayed in Swiss OF1 mice to determine the ED_{50} of the humoral response and antibody titers. The results show similar titers and ED_{50} in the three groups, which indicates the absence of interference by the different components of the DTP combination. They also show a satisfactory dose response of the antibody titers (Table 2). The ED_{50} values found are above 500 µg, i.e. five- to tenfold higher than those of PT in Swiss OF1 mice (50 to 150 ng). Therefore, if it is assumed that the protective contribution of these antigens in mouse models is related to antibody titers, it can be expected that the possible additive effects of PT and 69-kDa will appear only at the higher dosage of the latter.

(iii) Protective efficacy in mice. The purified 69-kDa protein was evaluated in the mouse intranasal and intracerebral challenge tests to assess its possible contribution to protective efficacy alone and in combination with the inactivated PT and FHA.

(iv) Intranasal challenge of mice. In the first intranasal test (Table 3A), in which 25-µg doses per animal were used, a high protection rate was obtained by PT alone or in combination with FHA and by the 69-kDa protein alone. Such high doses were inappropriate to evaluate a possible synergy of these different antigens in combination.

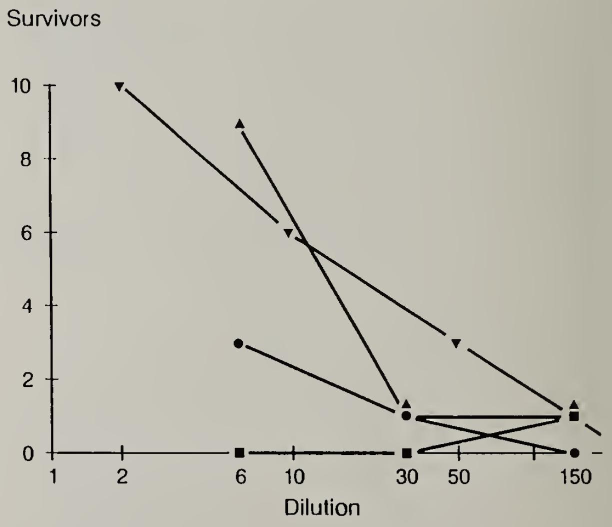

FIG. 7. Intracerebral challenge of mice. Mice immunized with dilutions of single doses containing: ■, 8 µg 69-kDa OMP; ●, 8 µg PT + 25 µg FHA; ▲, 8 µg 69-kDa OMP + 8 µg PT + 25 µg FHA; ▼, commercially available whole-cell DTP (Combivax).

In a second experiment (Table 3B), lower doses of each antigen were used. The results indicate a significant protection by 1.3 µg doses of 69-kDa protein: 7 out of 10 mice were fully protected, and the infected animals showed a much reduced bacterial count in the lungs. Association of the 69-kDa antigen with PT did not significantly modify this picture. In contrast, however, the addition of FHA to PT contributed to a significant reduction of the number of infected animals. This reduction was of course also observed in the three-component combination.

In the mouse lethal respiratory challenge, which uses a hypervirulent strain of *B. pertussis* which overproduces adenylate cyclase (1), complete protection was conferred by immunization with three 8-µg injections of the native or formalin-treated 69-kDa protein (data not shown).

Intracerebral challenge of mice. To explore the possible synergy between the 69-kDa OMP and the other acellular vaccine components PT and FHA, groups of mice were immunized with serial dilutions of a vaccine combining 8 µg of 69-kDa, 25 µg of FHA, and 8 µg of inactivated PT adsorbed to aluminum hydroxide. The mice were then challenged with the virulent strain 18323 as recommended by the European Pharmacopoeia.

The results show that the 69-kDa OMP component alone failed to confer any protection (Fig. 7). Combined PT and FHA gave an intermediate and dose-dependent protection which was inferior to that obtained with the whole-cell vaccine control. The three-component combination PT-FHA-69-kDa gave an improved protection rate similar to that obtained in the test by the whole-cell vaccine. This improvement over the two-component combination PT-FHA was apparent only at the higher vaccine dose: it was not observed at the intermediate and lower doses.

In another test, where successive dilutions of 69-kDa OMP were combined to 2.5 µg each of PT and FHA, a similar result was obtained (results not shown). The protective contribution of the 69-kDa OMP was obtained with 8- and 1.6-µg doses, but not with lower doses.

DISCUSSION

The gene of the 69-kDa OMP has revealed that this protein derived from a longer precursor by cleavage of the N-terminal signal peptide and removal of a C-terminal fragment (5). The latter cleavage site is as yet undetermined.

Isoelectric focusing of the purified 69-kDa protein shows two major species with pI 6.0 and 5.7. In addition, it has been found that the 69-kDa species is frequently converted to a form with an apparent molecular weight of 67 kDa. Eventually, the two species are co-purified and appear as a doublet in SDS-polyacrylamide gels (4). The identity of these different forms has been1 investigated more precisely by a combination of protein chemical studies.

The N-terminal sequence determinations of the different isoelectric forms of the protein confirmed the location of signal peptide cleavage. They did not identify any difference between the forms of pI 5.7 and 6.0. Similarly, the 67-kDa form also exhibited the same N-terminal sequence, implying that this form results from additional C-terminal cleavages.

Based on the observed molecular weight in SDS-gels, the C-terminal end has been tentatively located at the position Gly 677. Analysis of the C-terminal sequence of the purified 69-kDa protein and of the 67-kDa species was investigated by carboxypeptidase Y digestion, and by sequence determination and mass spectrometric studies performed on the intact proteins and on the C-terminal peptide obtained by limited trypsin hydrolysis.

The carboxypeptidase Y analysis excluded the C-terminal location at or near position 677. The composition of the amino acid mixture released indicated a possible location between residues 597 and 600. Due to the ambiguous identification of Ser and Asn, this experiment failed to give a more precise assignment.

The N-terminal sequence of the peptide resulting from limited trypsin cleavage at residue Arg 570 was compatible with a C-terminal location at Asn 597. The unambiguous confirmation of this position was obtained by accurate determination of the molecular size of the intact protein and its C-terminal tryptic peptide by ESMS. These determinations confirmed the predicted molecular weight of 60,282, which corresponds to the sequence extending from Asp 1 to Asn 597. In addition, the C-terminal tryptic peptide has an observed mass of 2,637; the predicted mass for the fragment Glu 571 to Asn 597 was calculated as 2,638. These experiments failed to yield information explaining the occurrence of forms of different pIs.

These data show that the actual size of the 69-kDa OMP is in fact much smaller than the apparent size determined by SDS gel electrophoresis. The occurrence of proline-rich repeated sequences in this protein (5) can explain this difference.

Similar methods were applied to investigate the naturally occurring cleavage to the 67-kDa form. In this case, ESMS on the whole protein indicated a mass of 58,132, consistent with a cleavage at Ala 575. These data, however, were partially obscured by the presence of Na^+ cations.

The sequence analysis of the C-terminal tryptic product gave a readable sequence to residue Asn 577. This discrepancy was cleared by ESMS analysis, which gave three signals corresponding to cleavages at residues 575, 576, and 577. These data are therefore indicative of a ragged C-terminus of the 67-kDa species, with cleavages at position Asn 577 and upstream.

Interest in the 69-kDa OMP as a potential vaccine component was initiated by the observation that the related 68-kDa OMP of *B. bronchiseptica* conferred protective immunity in susceptible animals (16). Study of the sera of whole-cell vaccine recipients or convalescents showed a strong immune response to the 69-kDa protein of *B. pertussis* (17, 19) which substantiates its interest as a human immunogen. Finally, it has been recently recognized that the 69-kDa OMP is present in significant amounts in Japanese acellular vaccines (17). This research therefore sought to demonstrate the innocuity, the immunogenicity, and the possible protective contribution in animal models of the purified 69-kDa protein in order to document its deliberate inclusion in vaccine formulations.

Tests in mice and newborn mice have demonstrated the absence of detectable contamination of the purified protein by other *B. pertussis* toxins as well as the absence of toxicity inherent to the 69-kDa OMP itself.

For experimental vaccine formulations, the mild formalin-amino acids treatment used for stabilizing FHA was applied. Like FHA, the 69-kDa protein appeared to be highly susceptible to proteolytic degradation, causing concerns about its long-term stability in vaccine preparations. Preliminary studies have indicated that, as in the case of FHA, this treatment does not reduce the immunogenic potential in laboratory animals.

In the intranasal challenge test in mice, the 69-kDa protein used alone as immunogen gave significant protection against lung colonization or in a lethal challenge using a hypervirulent *B. pertussis* strain. This effect, however, was not additive with that of PT and could not be demonstrated in combination with PT and FHA, since these antigens contribute to an almost complete protection. Future experiments should explore the possible additive effects of FHA and 69-kDa OMP in the absence of PT, since both factors are involved in bacterial adherence (4).

The intracerebral challenge with the PT-FHA combination showed a reduced protection rate compared to the whole-cell vaccine. The 69-kDa OMP increased the protection level to that of the whole-cell vaccine when administered at high doses. A steep decrease in protective level was observed at lower antigen dosage. This result is in line with the determination of the ED50 of the humoral immune response to the 69-kDa protein, which gave values of 0.5 to 0.7 µg — much higher than those of the other vaccine components, PT and FHA. Therefore, it is expected that the protective contribution of the 69-kDa component rolls off faster in serial vaccine dilution than that of PT and FHA themselves.

It is thus inferred that mice are possibly not an appropriate model for studying the interactions among the 69-kDa OMP, PT and FHA unless (i) specific strains of mice are used that respond equally to all these antigens or (ii) specific formulations with increased 69-kDa OMP dosage are prepared. These formulations are likely to differ significantly from the composition of the Japanese acellular vaccines (17) and of practically achievable formulations based on the recovery of the different antigens from *B. pertussis* cultures.

Given the strong immune response to relatively low doses of the antigen observed in human vaccine recipients, it can be suspected that the protective contribution of the 69-kDa antigen in humans may be quite different than that observed in mice. Only clinical studies involving deliberate addition of specific quantities of the pure antigen will contribute to evaluating this potential.

ACKNOWLEDGMENTS

This work was supported by the Région Wallonne, Ministère des Technologies Nouvelles.

We thank Anne Vaernewijck, Michèle Loiseau, Michael Huddleston, Nadine Pépin, and Carine Ysebaert for technical assistance and Martine Chevalier for typing the manuscript.

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DISCUSSION

DR. GOTTO: First I would like to say we have a poster this afternoon where we have shown many of the same observations and we have seen some of the same things. The question I have is do you observe naturally the 69-kDa form becoming the 67-kDa form without any treatment?

DR. CAPIAU: The purified protein shows one band at 69-kDa. Of course, sometimes we see a second band at 67-kDa. But normally we have only one band.

DR. GOTTO: Are these two slightly different molecular weight forms? Do they account for the two isoelectric forms they see?

DR. CAPIAU: No. The 69-kDa protein alone, the single band gives two bands on isoelectric focussing gels. Today I have no explanation for the two different forms that we observed in the isoelectric focussing gels. That may be. It is a (inaudible) in the protein, sometimes it happens between sequence asparagine-glycine and asparagine-serine and we have sequences like this in the protein.

DR. GOTTO: Yes, I do not explain this either but we do show that immunologically they are the same.

DR. CAPIAU: Yes, we too.

DR. GUPTA: You said that the 69-kDa protein is prone to break down to the 67-kDa protein. This 67-kDa protein is similar in immunogenicity and protection as the 69-kD protein?

DR. CAPIAU: We have not studied the immunogenicity of the 67-kDa product. We had only studied the biochemical analysis of the 67 but not the immunogenicity because we have preparations with one form, the 69.

DR. GUPTA: But during the production of the vaccine, if it is very prone to breakdown, then do you predict that in the end you may get 67-kDa protein?

DR. CAPIAU: No, we use only preparations with the 69-kDa species.

DR. RAPPUOLI: One of the reasons why the 69-kDa protein was originally brought forward for a vaccine was that it was described as improving the potency in the intracerebral challenge mediated by pertussis toxin alone. Now, I have not seen your data confirming that. We have tried to confirm that but we have not been able to see it. Have you tried to mix pertussis toxin or mutants of pertussis toxin plus 69-kDa and seen improvement?

DR. CAPIAU: As you have seen, we observed an improvement over the two component combination PT and FHA only at the higher vaccine dose but not at intermediate and lower doses. So we suspect that the mouse model is not appropriate to evaluate the synergy between these antigens unless a specific strain of mice is used or specific preparation was increased and 69-kDa doses are prepared. I think that is not possible for a vaccine candidate. Tests are ongoing in different labs and we are waiting for the results.

DR. QUENTIN-MILLET: We have also had similar results as those described by you. The only point that I would like to clarify -- I am not sure I got you straight -- is in our hands the immunogenicity, that is, the immune response of mice to purified 69-kDa is not at all that of 69-kDa present in whole cell. What I mean is we are getting much higher titers vaccinating with whole cell. And what this is telling us and I wonder if you have any comments, is that this is a membrane protein and as a lot of membrane proteins, if you take it out of its membrane environment the immunogenicity really drops.

DR. CAPIAU: We have observed exactly the same. You see the mouse model is maybe not appropriate and we need clinical studies to answer this question. I have no explanation for it. We are waiting for results in children and in infants.

DR. QUENTIN-MILLET: I think that this perhaps should be put into perspective of other outer membrane proteins, especially foreign proteins. For example, in *Neisseria gonorrhoeae* where results now are coming out to show that indeed the presentation of the protein to the immune system is of great importance. Maybe we should not be extracting these membrane proteins and using them as purified forms.

DR. CAPIAU: Well, wait and see.

SESSION TWO

Surface Proteins of *Bordetella pertussis:* Their Role as Bacterial Adhesins and Protective Antigens

Co-chairpersons:

Michael J. Brennan
James L. Cowell

Introduction

MICHAEL J. BRENNAN

This session is entitled Surface Proteins of *Bordetella pertussis*: Their Role as Bacterial Adhesins and Protective Antigens.

The first four talks are going to be involved with adhesion and entry of *Bordetella pertussis* into mammalian cells. The first speaker is David Relman and he will be talking about interactions of *Bordetella pertussis* with macrophages. The second speaker is Elizabeth Leininger who will be talking about the interactions of certain surface adhesins of *Bordetella pertussis* with mammalian cells.

So after we become attached to those two talks, we are going to be invaded by Mark Peppler followed by Cynthia Lee. They are going to talk about the entry of *B. pertussis* into mammalian cells. Then we will have Andy Robinson talk about fimbriae, Ian Charles talk about the 69-kDa or pertactin protein, I will talk about porin protein and we will end with Jan Poolman talking about surface proteins as protective antigens.

Mechanisms of *Bordetella pertussis* Adherence to Ciliated Respiratory Cells and Human Macrophages

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Whooping cough, or pertussis, results from infection by *Bordetella pertussis* and is the cause of substantial morbidity and mortality in many parts of the world. Humans are the only known natural hosts for this infection; in them, the primary site of infection is the respiratory tract. The key steps in pertussis pathogenesis are (i) entry by aerosol into a susceptible host, (ii) adherence to ciliated cells of the respiratory epithelium, (iii) avoidance or alteration of host natural immune defenses, (iv) multiplication and colonization of the respiratory tract mucosa, and (v) secretion of multiple toxins, leading to both local mucosal damage and systemic intoxication (20, 32). Observations of *B. pertussis* persistence within the respiratory tract and, in particular, within alveolar macrophages of experimentally infected animals (4, 7) raise interesting questions about the possibility of persistence within humans and the mechanisms by which this might occur. Recent studies suggest that *B. pertussis* may be capable of entering and surviving within some eukaryotic cells (6).

Adherence is a crucial event in pertussis pathogenesis, both for early colonization of the respiratory mucosal surface and for potential bacterial interactions with host immune cells. Two *B. pertussis* proteins, filamentous hemagglutinin (FHA) and pertussis toxin (PT), have been implicated as adhesins (29). Data are less clear concerning the roles of two other *B. pertussis* surface structures or proteins in adherence: fimbriae and pertactin (P.69 or 69 K). Previous efforts to determine the role of all of these factors in *B. pertussis* adherence have been hampered by the lack of detailed characterization of the relevant genetic loci. This paper presents and reviews recent data bearing on the role of FHA and PT in adherence to cilia and macrophages.

GENETICS OF *B. PERTUSSIS* ADHESIN EXPRESSION

Most, if not all, of the putative *B. pertussis* adherence factors are expressed under the positive coordinate control of a regulatory locus, the *bvg* operon (or *vir*) (1, 34). Control occurs at the level of transcription (15) and is directly mediated, at least in the case of FHA, by one component of this system, BvgA (24).

FHA is a surface-associated and secreted protein product which is unusually susceptible to breakdown into a number of polypeptide species (2, 10). The largest of these species, measuring approximately 220 kDa, has hemagglutinating activity and binds a number of eukaryotic cell lines in vitro (30). The structural gene for FHA, *fhaB*, lies adjacent to *bvg* (27) and is transcribed in the opposite direction (5). The complete nucleotide sequence of *fha*B is known (5, 22). Figure 1 illustrates some important features of this gene and the encoded gene product. The single 10774 base pair FHA ORF is far longer than that which would be required for expression of a 220-kDa polypeptide. Western immunoblot analysis using antisera generated against fusion proteins from various portions of the ORF suggests that the entire gene is translated (5). The mature product seems to be encoded by the 5′ portion of the ORF and appears after co- or post-translational proteolytic processing of a precursor polypeptide (Fig. 1). A specific Arginine-rich processing site (Arg-Arg-Ala-Arg-Arg, RRARR) has been identified by N-terminal amino acid sequence analysis of the 140-kDa and 125-kDa FHA polypeptide species (5). No function has yet been assigned to the Proline-rich C-terminus of the translated ORF, although predicted structural features suggest that it may serve as an anchor in the cell wall.

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* Presenting author.

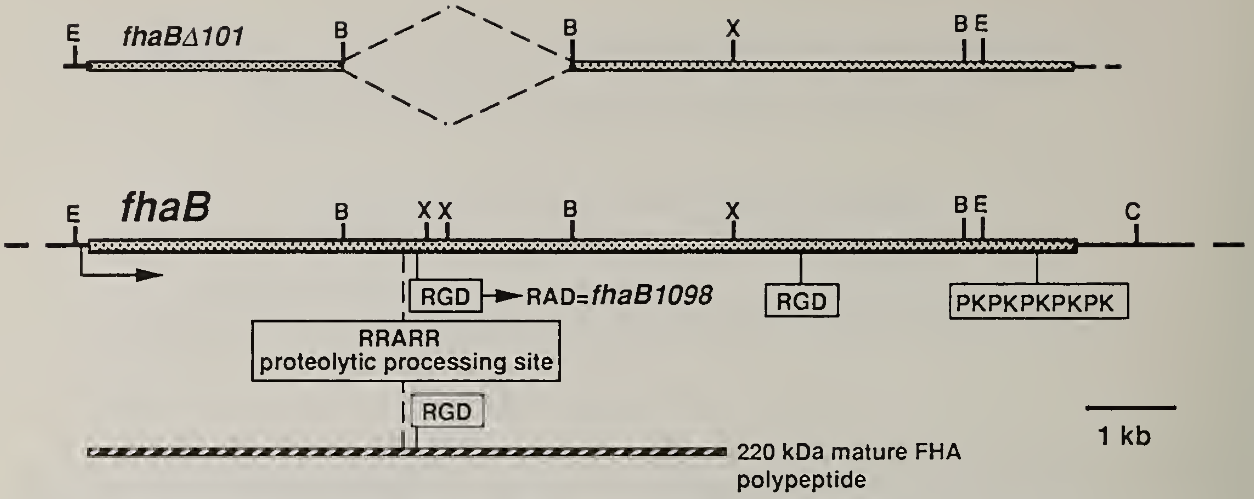

FIG. 1. A schematic map of the FHA structural gene *fhaB*. The location of the 10774 bp ORF is indicated by ▒. Deletion of an internal 2.4 kb *Bam*HI fragment (*fhaB*Δ101) leaves the gene in-frame and the protein product truncated. The portion of the ORF which encodes the mature FHA product is indicated by ▨. Significant features of the FHA-predicted amino acid sequence include the Arginine-rich processing site (RRARR), an alternating Proline-Lysine motif (PKPKPKPKPK), and two Arginine-Glycine-Aspartic acid sites (RGD). A site-directed mutation (*fhaB*1098) at the first RGD site leads to the substitution of Alanine for Glycine (RAD). E, *Eco*RI; B, *Bam*HI; X, *Xho*I; C, *Cla*I (not all *Cla*I sites are shown).

Within the predicted FHA peptide sequence are two Arg-Gly-Asp (RGD) tripeptide sites (Fig. 1). RGD sites are a common feature of a number of eukaryotic extracellular matrix proteins and mediate binding to the integrin family of heterodimeric protein receptors (9, 25). Only the first FHA RGD site is located in a portion of *fhaB* that is predicted to encode the mature 220-kDa, biologically active polypeptide (Fig. 1). In addition, peptide sequences flanking this first site share some homology with the functional fibronectin RGD site (22).

Pertussis toxin, a 105-kDa hexameric protein, acts systemically as an ADP ribosyltransferase with a wide variety of physiologic consequences for the host. It is encoded by the *ptx* operon which is unlinked to the *bvg* locus but which requires the latter for its regulated expression (8). The *ptx* operon has been sequenced and its regulatory sequences studied (13, 17). The 69-kDa outer membrane protein, pertactin, is an immunoprotective antigen in various animal studies (26) and may play a role in adherence. This protein is encoded by a gene whose length suggests that there is proteolytic processing of a precursor polypeptide (3), as in the case of FHA expression. In addition, the predicted amino acid sequence of pertactin also contains two RGD sites. The structural genes for serotypes 2, 3, and X fimbrial subunits also have been well characterized (12,16,19). *B. pertussis* fimbriae are antigenic in humans and experimental animals; however, their role in pathogenesis is unclear.

The above-described advances in the genetic characterization of *B. pertussis* virulence-associated genes allow a more precise analysis of adherence: isogenic strains can be constructed, each defective in one or more putative adhesins. These strains can then be studied for their binding activity using relevant eukaryotic target cells and tissue. Various *B. pertussis* BP536 derivatives (of the Tohama I and BP338 (34) lineage) were created for these kinds of adherence studies (Table 1). Results from subsequent experiments are described below.

RESULTS AND DISCUSSION

FHA is a dominant adhesin for *B. pertussis* adherence to ciliated respiratory cells. *B. pertussis* adherence to ciliated respiratory epithelial cells is dependent on the expression of *bvg*-regulated factors (29). Three of these factors have been studied through the creation of isogenic BP536 derivatives, and the results are concordant (22) (Table 2). BP101 expresses a truncated form of FHA which (i) is missing approximately 800 internal amino acid residues including the first RGD site and (ii) has no hemagglutinating activity. The entire *ptx* operon is deleted in strain BP-TOX6. In strain BP-B52, the *fim2* gene is deleted and the *fim3* gene is inactivated by a transposon insertion; therefore, no detectable fimbriae are made by this organism.

Table 2 summarizes data on the adherence of these strains (22). FHA is quantitatively the most important of

TABLE 1. *B. pertussis* strains

Strain[a]	Relevant genotype[a]	Relevant characteristics	Reference
BP536	*vir*$^+$ (*bvg*$^+$)	FHA$^+$, PT$^+$, Fim$^+$	22, 34
BP537	*vir*$^-$ (*bvg*$^-$)[b]	FHA$^-$, PT$^-$, Fim$^-$	22
BP-TOX6	*ptx*Δ6	PT$^-$	22
BP-B52[c]	*fim*2B52, *fim*3::Km	Fim$^-$	22
BP101	*fhaB*Δ101	ΔFHA[d]	22
BP101-TOX6	*fhaB*Δ101, *ptx*Δ6	ΔFHA[d], PT$^-$	23
BP1098	*fhaB*1098	FHA(RAD)[e]	23
BP1098-TOX6	*fhx*1098, *ptx*Δ6	FHA(RAD),[e] PT$^-$	23

[a] All strains are isogenic derivatives of BP536.
[b] Spontaneous phase variant.
[c] Gift from F. Mooi, RIVM, Bilthoven, The Netherlands.
[d] An internal, in-frame deletion in *fhaB* results in truncation of the mature FHA protein.
[e] The FHA RGD sequence at amino acid positions 1097 to 1099 is changed to RAD.

TABLE 2. Adherence of *B. pertussis* to ciliated respiratory epithelial cells and human macrophages in vitro

Strain	Relevant characteristics[a]	Levels of adherent bacteria (% relative to wild type, BP536)	
		Ciliated respiratory epithelial cells	Macrophages
BP536	FHA$^+$, PT$^+$, Fim$^+$	100	100
BP537	FHA$^-$, PT$^-$, Fim$^-$	0.0[b]	10[c]
BP-B52	Fim$^-$	94.2	179
BP101	ΔFHA	5.8[b]	105
BP-TOX6	PT$^-$	53.8	119
BP101-TOX6	ΔFHA, PT$^-$	0.0[b]	6[c]
BP1098	FHA(RAD)	87.3	137
BP1098-TOX6	FHA(RAD), PT$^-$	46.7[b]	42[c]

[a] See Table 1.
[b] The difference from BP536 is statistically significant at $P < 0.05$.
[c] The difference from BP536 is statistically significant at $P < 0.001$.

these three factors for binding to ciliated epithelial cells, but the absence of PT also leads to a significant decrease in binding. Fimbriae seem not to play an important role in adherence to ciliated cells in vitro. Chinese hamster ovary cells exhibit some of these same adherence requirements (22).

The mechanism(s) of FHA- and PT-mediated binding to ciliated cells is not entirely understood. It is known that FHA and PT have binding affinities for galactose and lactosamine-containing glycolipids on the cilia of human ciliated epithelial cells (28). PT lectin-like binding domains reside within B oligomer subunits (35). Current information indicates that the S2 subunit contains the galactose recognition site for cilia and S3 contains the sialic acid recognition site for macrophages (K. Saukkonen, N. Burnette, V. Mar, and E. Tuomanen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, D-136, p. 109). The precise FHA domains involved in the protein-carbohydrate interaction with cilia are unknown. The deletion in BP101 is too large to define a precise FHA-binding domain for ciliated cells, in part because the *fhaB*Δ101 mutation may alter global protein conformation.

The presence of an RGD site within the deleted portion of FHA in BP101 raises the possibility of an eukaryotic type of protein-protein binding. The role of this site in ciliary cell attachment has been studied using a strain, BP1098, which carries a site-directed mutation that replaces Glycine for Alanine (Table 1). BP1098 attaches to ciliated epithelial cells at 93% of the levels of its wild-type parent, BP536 (23). This result suggests either that this RGD site plays no role in binding to these cells or that this conservative mutation is not sufficient to cause a large enough change in adherence. Furthermore, it is possible that additional FHA residues at secondary sites participate in binding and can mask the effect of the *fhaB*1098 mutation.

These studies offer no direct conclusions on the role of pertactin in *B. pertussis* binding to ciliated cells or macrophages. The negligible binding of BP101-TOX6 in comparison to BP536 argues against a crucial role for factors other than FHA and PT; however, other workers have found that pertactin may mediate attachment to Chinese hamster ovary cells (Z. M. Li, E. Leininger, A. Bhargava, S. Stibitz, I. Charles, N. Fairweather, M. Roberts, P. Novotny, C. Manclark, and M. Brennan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, B-136, p. 49). Preliminary studies suggest that one of its RGD sequences may be involved (E. Leininger, J. G. Kenimer, C. A. Ewanowich, M. S. Peppler, and M. J. Brennan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, B-138, p. 49).

***B. pertussis* adheres to human macrophages using either FHA or PT.** As with ciliated cells, *B. pertussis* adherence to human macrophages is dependent upon the expression of *bvg*-regulated factors (23) (Table 2). BP537, a spontaneous Bvg$^-$ derivative of BP536, adheres to macrophages at 10% of the levels of its Bvg$^+$ parent. In contrast to the situation with ciliated cells, *B. pertussis* mutants deficient in a single virulence determinant — FHA, PT, or fimbriae — show no impairment in adherence to macrophages. However, a strain with a combination of FHA and PT mutations, BP101-TOX6, has markedly diminished binding, with levels indistinguishable from the Bvg$^-$ strain BP537 (Table 2). These data demonstrate that FHA and PT are each sufficient alone for maximal binding to macrophages, under the conditions defined in these assays (23).

Other combinations of mutations were not tested (e.g., fimbrial and FHA mutations); however, the similar adherence levels of BP101-TOX6, and BP537 suggest that *bvg*-dependent factors other than FHA and PT are not alone sufficient for *B. pertussis* binding to macrophages. It is important to note that BP101 does express a truncated FHA product. In interpreting the macrophage binding data using BP101, BP-TOX6, and BP101-TOX6, it is assumed that this truncated FHA product has no binding activity.

FHA is independently recognized by CR3 (CD11b/CD18) and a glycoconjugate on human macrophages. The mechanisms of FHA-mediated binding to macrophages were investigated further with the PT-deficient strain BP-TOX6 and some derivatives. Because FHA was known to adhere to galactose residues on ciliated cells, soluble galactose was used to block carbohydrate-dependent attachment of BP-TOX6 to macrophages. Binding of this strain was decreased by approximately 50% with soluble galactose; further inhibition was not achieved with concentrations of galactose as high as 50 mM (23). Other carbohydrates had less effect. Thus, both carbohydrate-dependent and -independent mechanisms seemed to be operative.

To study possible protein-protein mediated binding, human macrophages were plated on substrates coated with a variety of monoclonal antibodies against known macrophage surface proteins (23). In this assay these antibodies cause their respective ligands to migrate to the substrate-adherent portion of the macrophage, thereby depleting the apical surface of that specific receptor (36, 38) (Fig. 2.) In the presence of 50 mM of galactose, antibodies to either the α or β chain of CR3 caused a decrease in binding of BP-TOX6 to less than 40% of control values. Antibodies to the α chains of the other two β_2 integrins or to other macrophage constituents had no effect. To confirm that CR3 acts as a receptor for FHA, a CR3$^+$ macrophage-like cell line THP-1 was tested for binding to human serum albumin, fibronectin, fibrinogen, and FHA. As expected, these cells adhered to the known CR3 ligand, fibrinogen, as well as to FHA

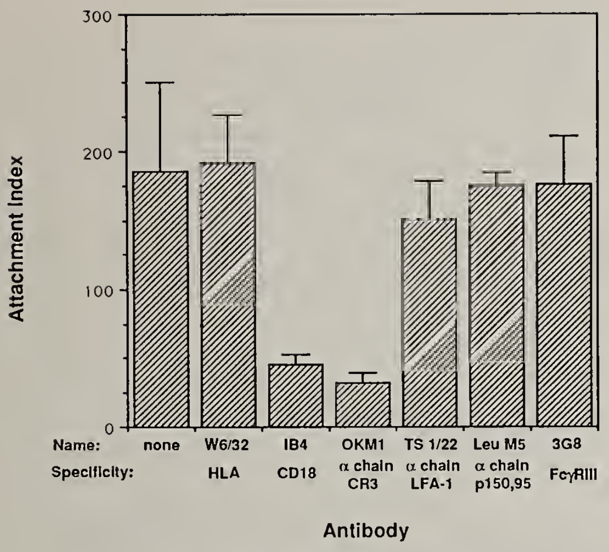

FIG. 2. Effect of antibodies on the adherence of *B. pertussis* BP-TOX6 to human macrophages. Antibodies on tissue culture well surfaces caused migration of their respective ligands to the adherent surface of bound macrophages. The apical macrophage surface becomes depleted of these ligands, or receptors. The attachment of BP-TOX6 was measured in the presence of 50 mM of soluble galactose (23). The attachment index was defined as the mean number of adherent bacteria on 100 macrophages. The error bars indicate standard deviation about the mean.

(23) (Fig. 3). A variant of THP-1 deficient in the β_2 integrins bound significantly less well to both of these proteins. THP-1 and its variant attached to fibronectin equally well; fibronectin is not a ligand for CR3.

Many protein ligands are recognized by integrins via an RGD sequence. Two approaches have been used to investigate the possible role of the first FHA RGD site in recognition by CR3 (23). The first involves the study of the site-directed mutation *fhaB*1098. This RGD mutation had no effect on expression of full-length FHA protein. In the absence of PT, this conservative mutation decreased adherence to macrophages to 42% of the level of BP536 (Table 2). In the presence of galactose, the RGD mutation drops binding of this strain by 80% (23).

The role of the first FHA RGD sequence in binding to macrophages was confirmed with a second experimental approach. Synthetic nonameric peptides encompassing this portion of *fhaB* (residues 1094 to 1102) were tested for their ability to inhibit *B. pertussis* binding. Peptide TVG*RGD*PHQ blocked binding of BP-TOX6 to macrophages by more than 90% in the presence of soluble galactose (23). A control peptide, TVG*RAD*PHQ, caused no inhibition.

What are the possible results of recognition of FHA by CR3? First of all, FHA-mediated binding to CR3 may facilitate entry of *B. pertussis* into human macrophages. Binding to CR3 may elicit phagocytic engulfment but avoid the induction of a macrophage respiratory burst (37). Although there is no evidence that *B. pertussis* survives within macrophages during natural human infection, studies of murine pertussis infection have suggested that organisms can be found inside alveolar macrophages (4). Macrophages might serve as a reservoir for persistence of *B. pertussis* in the host. As a second possibility, FHA-mediated binding to CR3 may provide a means for delivery of *B. pertussis* toxins such as PT and adenylate cyclase to macrophages. Both of these molecules have the ability to modify the function of host immune cells (14, 18). Third, excreted FHA may saturate CR3 receptors in the local environment, thereby blocking normal CR3 interactions. More than one of these possibilities may be operative during human infection by *B. pertussis*.

***B. pertussis* adherence in the natural host.** In nature, *B. pertussis* infects only humans. Therefore, from a theoretical point of view, the only relevant methods for studying *B. pertussis* adherence are either with an intact human host or with human tissues and cells normally exposed to this organism during natural infection. These

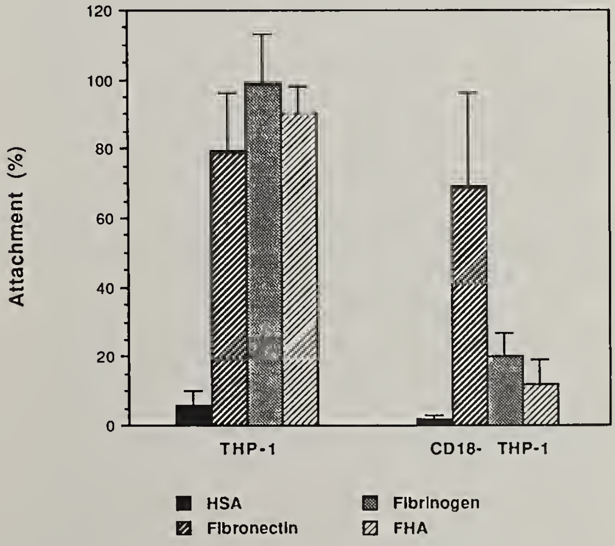

FIG 3. Attachment of macrophage-like THP-1 cells to various proteins. Binding of THP-1 cells to each of the four indicated proteins is compared to the binding of a CD18-deficient variant of THP-1 (23). Attachment of cells is expressed as a percentage relative to the total number of cells added. The error bars indicate standard deviation about the mean.

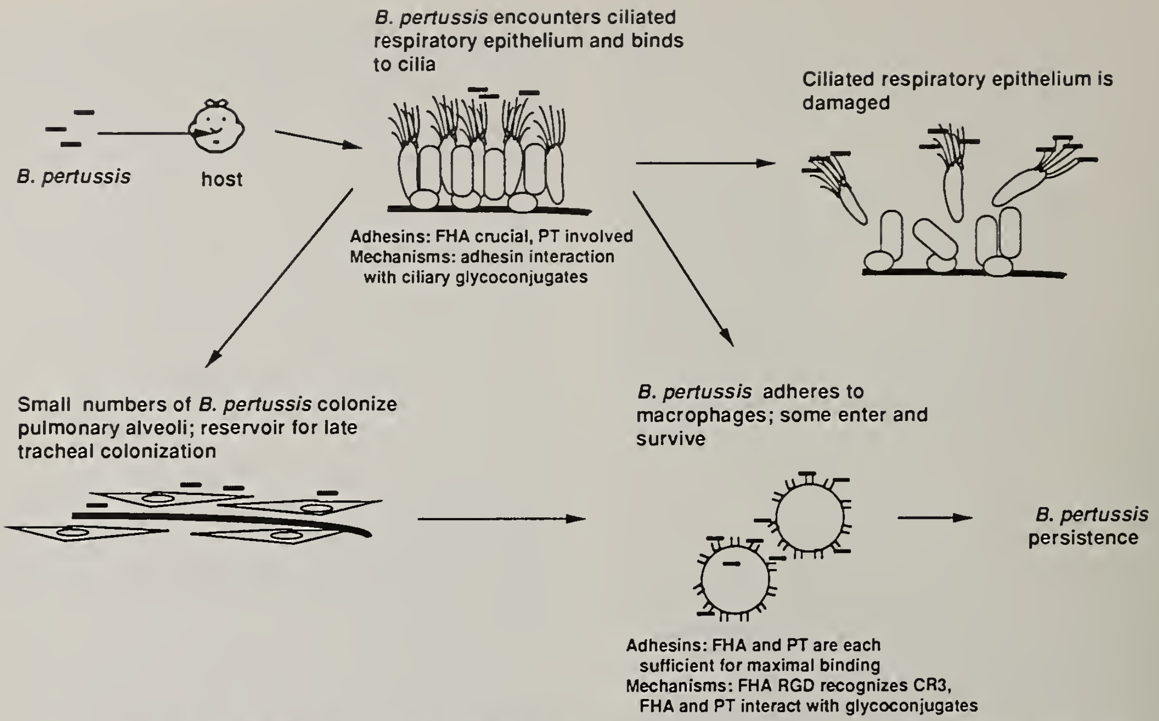
B. pertussis encounters ciliated respiratory epithelium and binds to cilia
B. pertussis
host
Ciliated respiratory epithelium is damaged
Adhesins: FHA crucial, PT involved
Mechanisms: adhesin interaction with ciliary glycoconjugates
Small numbers of B. pertussis colonize pulmonary alveoli; reservoir for late tracheal colonization
B. pertussis adheres to macrophages; some enter and survive
B. pertussis persistence
Adhesins: FHA and PT are each sufficient for maximal binding
Mechanisms: FHA RGD recognizes CR3, FHA and PT interact with glycoconjugates

FIG 4. A hypothetical scheme indicating *B. pertussis* adherence interactions during the course of natural infection. Adhesins and attachment mechanisms relevant to these interactions are indicated where there is information available. Because data from in vivo experiments are sparse, some statements are highly speculative.

considerations impose substantial constraints. While human upper respiratory mucosal tissue may be available occasionally, harvested respiratory epithelial cells and immune cells are the most practical human adherence targets for *B. pertussis* investigations. The results presented above involving the use of ciliated cells and macrophages are most relevant to pertussis pathogenesis. On the other hand, if their limitations are acknowledged, animal models also can offer useful information on *B. pertussis* virulence and colonization (21).

The infant mouse respiratory infection model of pertussis has provided some insight into attachment mechanisms. Studies with death as an endpoint have suggested that FHA is not crucial for virulence (31, 33). Because death is usually secondary to pneumonia, it is not surprising that colonization of the lung in these studies appears not to be affected by transposon insertions within FHA regulatory or structural loci. When colonization of a more relevant respiratory tissue, the tracheal mucosa, is examined in a nonlethal aerosol infection model, FHA appears to be an important factor (11). BP101, the FHA deletion mutant, is cleared from the trachea early in infection but recolonizes this tissue at later time points. BP536, the wild-type parent strain, colonizes the trachea in substantial numbers throughout the test period. At the same time, in this study BP101 colonizes the lung as well as the wild-type parent (11). These results are consistent with the data presented earlier demonstrating the importance of FHA in ciliary cell attachment. One possible explanation for the recolonization of the trachea by BP101 at later time points is that the tracheal surface becomes damaged during the early phases of infection. A damaged or altered tracheal epithelium may present new surfaces and receptors for BP101 attachment, mediated by binding domains found in the truncated form of FHA or in other adhesins.

Figure 4 summarizes the current understanding of the adhesins and mechanisms used for *B. pertussis* attachment to relevant targets and offers a hypothetical scheme for the role of different binding interactions during natural infection. A number of issues are now under study. The lectin-like adherence mechanisms for FHA and PT which have been demonstrated in vitro seem to be operative in vivo since competitive carbohydrates (receptor analogs) and antireceptor antibodies block colonization of the lung in a rabbit model (K. Saukkonen and E.

Tuomanen, Abstr. 1990 World Conference on Lung Health). The first FHA RGD site appears to be important in lung colonization and bacterial uptake into bronchoalveolar macrophages in the rabbit model (K. Saukkonen, C. Cabellos, M. Burroughs, and E. Tuomanen, Abstr. Microbial Adhesion and Invasion 1990). In addition, this FHA RGD site appears to be involved in colonization of the mouse trachea (D. Relman, A. Kimura, K. Mountzouros, S. Falkow, and J. Cowell, unpublished data). These results will require confirmation and extension to define the relative roles of adherence to different target cells within the respiratory tract. Possible consequences of *B. pertussis* interaction with macrophage CR3 are discussed above. If entry into and survival within macrophages does occur in vivo, then the first RGD sequence may indirectly affect *B. pertussis* persistence within the host lung or upper respiratory tract. It can be speculated that this RGD-CR3 interaction may take place at the lymphoid follicles within the nasopharyngeal mucosa, leading to bacterial persistence.

The significance of the second FHA RGD site and the FHA C-terminal sequences for adherence are unknown. Further mutational analysis will be necessary to map other binding domains within this molecule as well as within other *B. pertussis* adhesins. An understanding of *B. pertussis* adherence at the molecular level will likely lead to improved therapeutic and prophylactic strategies for pertussis.

ACKNOWLEDGMENTS

This work was supported in part by the following grants: a Bank of America-Giannini Foundation Medical Research Fellowship, a Daland Fellowship of the American Philosophical Society, and a Lucille P. Markey Scholar Award in Biomedical Science to D.A.R.; National Insitute of Allergy and Infectious Disease (NIAID) grant 23459, the I. T. Hirschl Trust, and a Career Investigatorship of the American Lung Association to E.T.; NIAID grant 23945 and a generous gift from Praxis Biologics, Inc., to S.F.; NIAID grants 22003 and 24775 and an Established Investigatorship of the American Heart Association to S.D.W.

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DISCUSSION

DR. MASURE: You spent a lot of time looking at the nature of the attachment of FHA to macrophages and from what I understand, FHA is processed such that there are many different forms. I wonder if you could speculate on the nature of the attachment of FHA to the bacteria.

DR. RELMAN: FHA, as some of you may know, can act as an exogenous protein and bind a number of bacteria, some of which have been studied by Elaine Tuomanen, namely pneumococcus and I believe *Haemophilus influenzae* but in addition, FHA is known to be a self-aggregating protein and FHA can coat *Bordetella pertussis* strains either bearing their own FHA or strains that are devoid of FHA. As far as the mechanism for FHA protein interactions with other bacteria as well as itself, I think it is probably in large part not understood. The protein itself is overall very hydrophobic and there may be a number of non-specific hydrophobic interactions that mediate FHA-FHA binding but the interactions of FHA with a foreign surface are not clear.

DR. LOCHT: I noticed that with your RGD mutant together with the toxin minus strain, you had a decrease in macrophage binding and also in ciliated cell binding down to the about the same level, 46 percent. So do you think there is an integrin FHA interaction for ciliated cells?

DR. RELMAN: You are right, although in that table the binding of the toxin deletion mutant alone, *tox*-6, was roughly the same as the binding of the double mutant; the RGD mutation plus the toxin mutation. So an independent separate role for that RGD mutation is not apparent from those studies on ciliated cell binding. Whether in separate studies in an intact animal the RGD site may be involved is somewhat not known at the present time. There are preliminary data that we have that the RGD site may be involved in tracheal colonization, but whether that is the same process that we are looking at in the test tube or in vitro is not clear.

DR. PEPPLER: Just for perspective, we used to hear a lot about FHA interacting with cholesterol and nowadays we do not at all. I was wondering if there is, in fact, a binding site for cholesterol on FHA, do you know what it is, and what are the relative affinities, if that is known, for cholesterol versus some of the sugar or sugar substrates for FHA?

DR. RELMAN: I am not sure I can comment directly on a potential cholesterol interaction. If FHA is like other eukaryotic extracellular matrix proteins, it very probably does have a number of different binding domains for perhaps eukaryotic proteins, the way fibronectin does with fibrin and collagen, etc. Whether FHA has a domain for a cholesterol-like interaction is entirely possible. I do not have any direct data to support that. Maybe Elaine Tuomanen has some comments on that.

DR. TUOMANEN: I do not have anything to comment on cholesterol but perhaps it might help to talk about some of the preliminary data that you have mentioned in terms of the role for this interaction in vivo. Looking at the rabbit model, we have been able to do broncho-alveolar lavage on these animals and at 24 hours of infection, approximately half the population of *Bordetella* in the lung is recoverable from macrophages. So that probably is a significant proportion of the total bacterial load within the lung.

However, the mechanism by which they get into macrophages appears to be exclusively by the RGD interaction and not by the carbohydrate-mediated types of interactions. But interestingly, the only way you can prevent pulmonary pathology is by blocking the carbohydrate dependent interaction. This has led us to hypothesize that perhaps the bacteria that are within the macrophages are not inducing the pulmonary pathology, either because the toxins are sequestered or they are not produced or other possibilities. We would suggest that the phenomenon that you have just described is a fairly significant one in terms of augmenting the bacterial load during the process, at least early in the course of colonization/disease.

Surface Proteins of *Bordetella pertussis:* Role in Adherence

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The interaction of the human pathogen *Bordetella pertussis* with host cells, an event that leads to colonization and subsequent infection, appears to occur through a complex mechanism involving various bacterial proteins, some with multiple binding sites recognizing different mammalian cell receptors. This use of various attachment proteins is obviously advantageous to the bacterium, as it confers an ability to bind to different cells depending on physiological conditions and localization. Until recently, *B. pertussis* has been stereotyped as a noninvasive bacterium that only colonizes the ciliated epithelia. However, in 1989, Ewanowich et al. found that *B. pertussis* could invade and survive within tissue culture cells (3). In vitro, *B. pertussis* can interact with a variety of cells including ciliated cells (13), WiDr cells (15), Hela cells (3) and macrophages (8). Therefore, it seems reasonable to speculate that various proteins acting independently or synergistically are involved in the interaction of *B. pertussis* with mammalian cells.

Filamentous hemagglutin (FHA) has been identified as a cell adhesin of *B. pertussis*. This 220-kDa protein is found on the surface of the bacteria and interacts with red blood cells, thereby causing hemagglutination. Mutants lacking FHA expression show a decrease in attachment to WiDr cells (15) and ciliated cells (7). Pertussis toxin has also been shown to affect the adherence of *B. pertussis* to ciliated cells (13). When neither FHA nor pertussis toxin are expressed, the attachment of *B. pertussis* to macrophages is decreased (8). Furthermore, the interaction of FHA with macrophages seems to involve the sequence Arginine-Glycine-Aspartic acid (RGD) as shown with an FHA site-specific mutant which also does not express pertussis toxin (8).

A 69-kDa protein has been identified on the surface of virulent strains of *B. pertussis* (1,6). Antibodies specific for this molecule are found in humans following disease or vaccination with whole-cell pertussis vaccine (12). Animal studies have demonstrated that this protein is a protective antigen (10) that induces the production of antibodies that can agglutinate virulent *B. pertussis* cells (For these reasons this protein is being evaluated in ew acellular vaccines for whooping cough. This report provides evidence that the 69-kDa protein can attach to mammalian cells and may also participate in the complex interaction of *B. pertussis* with host cells. Since this is the first functional role suggested for the protein, the name "pertactin" has been proposed to illustrate the *tac*tile role of this *per*tussis protein. In the studies presented here, the interaction of pertactin with eukaryotic cells is compared with that of FHA.

MATERIALS AND METHODS

Materials. The 69-kDa surface protein, pertactin, was a gift from Pavel Novotny (Wellcome Biotech, Kent, England). FHA was obtained from Pasteur Merieux Serums et Vaccins-Marcy l'Etoile-France. Monoclonal antibodies BPE3 and BPD8 which react with pertactin (1), and the antifimbrial monoclonal antibody BPF2 (5) have been described previously. The monoclonal antibody MO8-X3C, directed against FHA, will be described elsewhere (E. Leininger, P. G. Probst, J. L. Cowell, and J. G. Kenimer, unpublished data). Chinese hamster ovary (CHO) cells (ATCC CCL61), HeLa cells (ATCC CCL2) and P388.D1 cells (ATCC TIB63) were purchased from American Type Tissue Collection; CHO 15B cells were a gift from Nathan Sharon. The structural change in the glycoproteins expressed by the CHO 15B cell variant has been described previously (4). [^{3}H]-thymidine (6.7 Ci/mmole) was supplied by Dupont-New England Nuclear, and L-[35S]-methionine (1,160 Ci/mmole) was obtained from Amersham Corp., Arlington Heights, Ill.

Peptides. Peptides, corresponding to RGD-containing sequences in pertactin or FHA, were synthesized using an Applied Biosystems 430A peptide synthesizer. These peptides included i) a 14 amino acid peptide

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* Presenting author.

TABLE 1. Attachment of CHO cells to pertactin and FHA

	Pertactin (cpm)	FHA (cpm)
Monoclonal Antibody		
Control	47,601 ± 4,551	28,763 ± 5,379
MO8-X3E (anti-FHA)	na	13,217 ± 1,788
BPE3 (anti-pertactin)	7,647 ± 730	na
Peptide		
Control	20,375 ± 1,323	26,769 ± 3,601
PRN-1-RGD	4,280 ± 410	24,904 ± 5,396
PRN-3-RGE	16,505 ± 1,126	41,264 ± 13,852
FHA-1-RGD	17,880 ± 1,187	28,665 ± 3,246

Pertactin and FHA were coated on 96-well microtiter plates, each at a concentration of 30 µg/ml. Chinese hamster ovary cells were labeled overnight with [^{3}H]-thymidine as described in text. All peptides were used at a final concentration of 1 mg/ml. Purified monoclonal antibodies were used at a protein concentration of 1 mg/ml. The controls contained no monoclonal antibody or peptide, respectively.
na: Not available.

corresponding to the sequence containing the first pertactin RGD, ATIRR**RGD**ALAGGAC (PRN1-RGD); ii) the corresponding control peptide ATIRRGEALAGGAC (PRN3-RGE); and iii) a 14 amino acid peptide corresponding to the sequence containing the first FHA RGD, VTVG**RGD**PHQGVLC (FHA1-RGD). Purity of the peptides was verified by high-pressure liquid chromatography. The carboxy-terminal Cysteine residue on the peptides was added to facilitate peptide conjugation and immobilization. These peptides had no visible effects on the mammalian cells during the course of the inhibition assay as determined by staining and microscopic examination.

Cell attachment assays. Chinese hamster ovary (CHO) cells cultured in HAMS-F12 media (S&S Media, Rockville, Md.) supplemented with 2 mM of Glutamine, 20 µg/ml of gentamicin (Flow Laboratories, Inc., McLean, Va.), and 10% fetal calf serum (HyClone, Logan, Utah) were labeled overnight with [^{3}H]-thymidine (New England Nuclear Corp., Boston, Mass., 6.7 Ci/mmol), 25 µCi per 75 cm^2 flask. The cell attachment assay was essentially performed as described by Ruoslahti et al. (9). In brief, 96-well microtiter plates (Costar, Cambridge, Mass., #3590) were coated with 30 µg/ml of pertactin or FHA (60 µl/well) in Dulbecco's phosphate-buffered saline for 3 h at room temperature and blocked with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo. A7638) in Dulbecco's phosphate-buffered saline for 1 h. One hundred µl of attachment buffer (HAMS-F12, 2 mM of Glutamine, 20 µg/ml of gentamicin, and 2 mg/ml of bovine serum albumin) was added to each well followed by 100 µl of [^{3}H]-thymidine labeled CHO cells at 5 x 10^5 cells/ml. The plates were incubated for 60 min at 37°C and then the wells were washed. Cells which remained attached after two washings were solubilized with 10% sodium dodecyl sulfate and radioactivity was counted in a Beckman liquid scintillation counter. Peptide or monoclonal antibody inhibition of CHO cell attachment to pertactin or FHA was assessed by adding the peptides or purified monoclonal antibodies to the microtiter plate assay wells immediately before adding the [^{3}H]-thymidine labeled CHO cells. Peptides and monoclonal antibodies were at a final concentration of 1 mg/ml. The results presented in Table 1 are the means of triplicate determinations and each experiment was repeated at least two times.

Bacteria. *B. pertussis* 18323 and Tohama I were grown as described previously (1) using Bordet-Gengou agar medium (Difco Laboratories, Detroit, Mich.) containing 15% defibrinated rabbit blood. This growth was used to inoculate flasks containing liquid Cohen-Wheeler medium (S&S Media); the culture was continued for 24 to 36 h with constant agitation in the presence of 25 µCi/ml of L-[^{35}S]-methionine (Amersham, 1,160 Ci/mmole). The specific activity of the radiolabeled bacteria was approximately 10^{-4} cpm per organism. *B.*

pertussis mutants deficient in the expression of pertactin (BBC16; -PRN), FHA (BBC17; -FHA), or both proteins (BBC18; -PRN,-FHA) were constructed from an 18323 streptomycin-resistant strain (BBC15) and will be described elsewhere (Roberts et al.; Brennan et al.). To characterize these mutants, bacterial extracts were examined using a variety of techniques including immunoblots, CHO cell assay, hemagglutination, assays for adenylate cyclase activity, and bacterial agglutination.

Bacterial attachment assay. Confluent monolayers of CHO, HeLa, or P388.D1 cells in 96-well tissue culture plates (Costar) were washed three times with attachment buffer (Hanks balanced salt solution [Flow Laboratories] containing calcium and magnesium and 0.2% bovine serum albumin). [^{35}S]-labeled *B. pertussis* cells (40,000 cpm per well) were added to the wells and incubated for various periods of time at room temperature; the wells were washed three times with attachment buffer. To solubilize the attached bacteria, 10% SDS was added to each well. The samples were then counted in a Beckman scintillation counter after dissolving in Hydrofluor (National Diagnostics, Manville, N.J.). The results shown in Figs. 1 and 2 are the means of triplicate determinations; and wells were scanned using phase optics to verify that the bacteria were bound to intact cell monolayers.

RESULTS

Radiolabeled CHO cells attach to plastic wells coated with 30 µg/ml of either purified pertactin or FHA (Table 1). The specificity of attachment was assessed using bovine serum albumin, which does not promote cell attachment. Monoclonal antibodies reactive with either pertactin or FHA specifically inhibit cell attachment to their respective antigens. Monoclonal antibody BPE3 directed against pertactin inhibits attachment of CHO cells to pertactin by 84%; monoclonal antibody MO8-X3E reactive with FHA blocks attachment to FHA by 54% (Table 1). Neither of these antibodies had any effect on the attachment of CHO cells to fibronectin, a well-known mammalian cell adhesion protein (data not shown).

Recent cloning and sequencing of the genes for pertactin and FHA revealed the presence of RGD sequences in both proteins (2, 7). This amino acid sequence has been shown to function as a cell-binding site present in a number of eukaryotic cell attachment proteins. RGD-containing peptides 14 amino acids in length were synthesized corresponding to the RGD sequences in pertactin (PRN1-RGD) or FHA (FHA1-RGD). A peptide containing a substitution of Glutamic acid for Aspartic acid in the pertactin peptide was used as a control (PRN3-RGE). (See Materials and Methods for complete sequence.) The RGD peptide from pertactin at a concentration of 1 mg/ml inhibited the attachment of CHO cells to pertactin by 79% (Table 1). The RGD peptide from FHA and the control peptide had no significant effect on attachment of the cells to pertactin. None of the peptides had an effect on the attachment of CHO cells to FHA (Table 1).

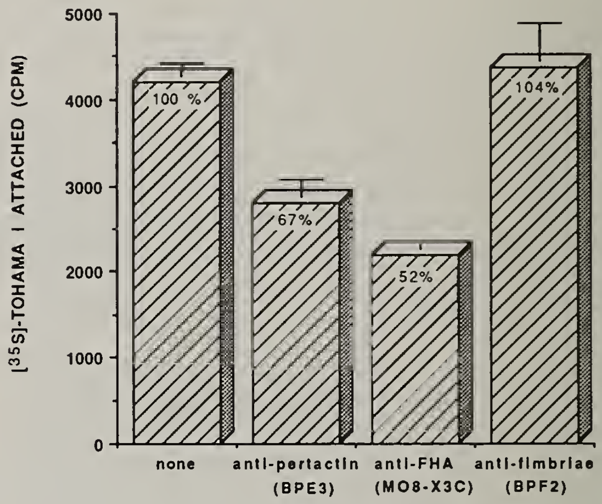

FIG. 1. Effect of monoclonal antibodies on the attachment of *B. pertussis* Tohama I to CHO cell monolayers. The [^{35}S]-labeled bacteria (2 x 10^9 organisms/ml) were preincubated for 30 min with 100 µg/ml each of purified monoclonal antibodies directed against pertactin (BPE3), FHA (MO8-X3C), or type 2 fimbriae (BPF2). Bacteria (40,000 cpm/well) were then added to confluent monolayers of CHO cells in 96-well plates for 4 h, and attached bacteria were measured as described in text. The average counts per minute of three wells with the standard deviations are shown as well as the percentage of attachment compared to the no-antibody control.

Since the RGD site in FHA appears not to be interacting with CHO cells and there is evidence that FHA may interact with cells through a lectin-like mechanism, the ability of a CHO cell variant (CHO 15B) to attach to FHA was assessed. CHO 15B cells, which contain altered surface glycoproteins, attached 25% better to purified FHA than did the wild-type CHO cells (data not shown).

To determine if monoclonal antibodies directed against pertactin, FHA, or type 2 fimbriae inhibit the attachment of bacteria to mammalian cells, a wild-type strain of *B. pertussis*, Tohama I, which expresses all three of these antigens was radiolabeled and incubated with

CHO cells in the presence of these antibodies (Fig. 1). The anti-pertactin antibody BPE3 inhibited bacterial attachment by 33% and the anti-FHA antibody MO8-X3C inhibited attachment by 48%. This finding suggests that these two proteins are involved in adherence. The anti-fimbriae monoclonal antibody BPF2 had no effect on attachment.

Mutants lacking the expression of these proteins were constructed in *B. pertussis* strain 18323 using the vector pRTP1 (11) to further investigate the possible role of the proteins in attachment. The bacteria were radiolabeled and incubated with CHO cell monolayers (Fig. 2). Compared to the streptomycin-resistant parent strain BBC15, the attachment of the pertactin-minus mutant BBC16 was decreased by 28%, the attachment of the FHA-minus mutant BBC17 was decreased by 36%, and the attachment of the double mutant BBC18 (-PRN,-FHA) was decreased by 63%. Similar results were observed when these bacterial strains were incubated with human HeLa cells or mouse P388.D1 macrophages. BBC16 (-PRN) attached 49% less well to HeLa cells and 33% less well to the macrophages. BBC17 (-FHA) adhered 56% less well to HeLa cells and 31% less well to the P388.D1 cells. The double mutant BBC18 (-PRN,-FHA) attached 79% less well to HeLa cells and 62% less well to macrophages, suggesting a synergistic effect of the two mutations.

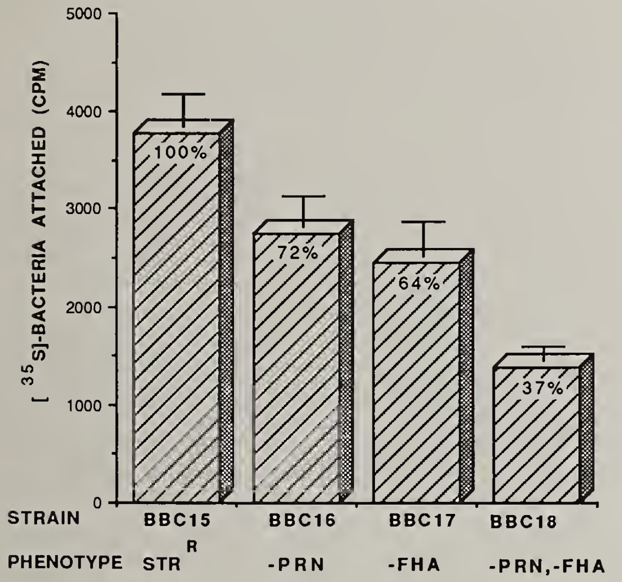

FIG. 2. Adherence of *B. pertussis* 18323 mutants to CHO cells. [^{35}S]-labeled bacteria (40,000 cpm/well) were incubated with monolayers of CHO cells for 8 h, and the adherent bacteria were measured as described in text. The average counts per minute and standard deviation obtained in three wells and the percentage of attachment compared to the BBC15 parent strain are shown.

DISCUSSION

B. pertussis expresses various proteins on its surface that may be involved in bacterial attachment to mammalian cells. The role of the purified surface proteins pertactin and FHA in promoting the adherence of mammalian cells has been investigated. Substrates coated with either pertactin or FHA can support the adherence of CHO cells and other cultured cells in a manner similar to that previously seen with eukaryotic cell adhesion proteins such as fibronectin. The specificity of this cell binding is demonstrated by the ability of monoclonal antibodies directed against pertactin or FHA to inhibit attachment of cells to the appropriate bacterial protein. Also, like fibronectin, both of these bacterial proteins contain RGD sequences which have been shown to function as cell-binding sites in eukaryotic cell attachment proteins. An RGD-containing peptide constructed from the sequence of pertactin (PRN-1-RGD) can inhibit the attachment of CHO cells to pertactin—but not to FHA-coated substrates. Substitution of the Aspartic acid with Glutamic acid (PRN-3-RGE) destroys the ability of this peptide to inhibit attachment. These findings indicate that the RGD sequence is important in the interaction of mammalian cells with pertactin. The RGD peptide synthesized from the FHA sequence (FHA-1-RGD) did not block the adherence of CHO cells to FHA or pertactin, suggesting that the interaction of CHO cells with purified FHA is not mediated through the RGD site. This idea contrasts with recent evidence that indicates that the FHA-mediated interaction of *B. pertussis* with human macrophages can occur through an RGD site. Human macrophages appear to be expressing a receptor (CR3) in the appopiate conformation capable of interacting with the RGD sequence of FHA (8). The results of the present study demonstrate that CHO cells which contain altered surface carbohydrates associated with membrane glycoproteins attach differently to FHA, suggesting that the attachment of CHO cells to FHA may occur through a lectin-like binding site on FHA. The mechanism of attachment of CHO cells to FHA may be more like that suggested for the interaction of *B. pertussis* with cilia (14). Evidence for a lectin-like binding site on FHA is further supported by the ability of FHA to agglutinate red blood cells.

The importance of pertactin and FHA in mediating adherence of the whole bacteria was further investigated by assaying the attachment of the bacteria to cell monolayers. A monoclonal antibody (BPE3) reactive with pertactin blocked adherence of *B. pertussis* Tohama I to CHO cells by 33%, and a monoclonal antibody directed against FHA (MO8-X3C) blocked attachment by 48%, suggesting that the adherence of bacterial cells to CHO cells is mediated at least in part by both pertactin and FHA. This hypothesis is supported by the finding that mutants deficient in the expression of pertactin, FHA, or both attach less well to CHO cells. Similar results were obtained with HeLa cells and mouse macrophages. Although some differences among mammalian cell types were observed, the pertactin mutant adhered at least 30% less well than the parent strain, the FHA mutant about 40% less well, and the mutant deficient in the expression of both proteins about 60% less well. Although these results suggest that both proteins are participating in adherence, they also suggest that other adhesins may be contributing to the bacterial interaction with the mammalian cells. Results using monoclonal antibodies directed against *B. pertussis* fimbriae and mutants lacking fimbriae (unpublished results) suggest that fimbriae play no role in attachment. The additional attachment could be accounted for by pertussis toxin or other surface components; this premise is currently under investigation.

ACKNOWLEDGMENTS

We thank Pavel Novotny for purifed pertactin; and Ian Charles, Mark Roberts, and Neil Fairweather from Wellcome Biotech and Zhong Ming Li, Scott Stibitz and Amit Bhargava at the Center for Biologics Evaluation and Research for their contributions to the construction and characterization of the bacterial mutants. We are also grateful to Kenneth Seamon and Judy Regan at the Laboratory of Molecular Pharmacology, Center for Biologics Evaluation and Research for the synthesis of the peptides used in this study.

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DISCUSSION

DR. PARKER: Did you try to inhibit binding using an anti-pertussis toxin monoclonal and if so, what were the results?

DR. LEININGER: Yes, but unfortunately at the time I had an antibody directed against the S-1 subunit and, of course, there was no inhibition. I need to try with antibodies directed against the B oligomer.

DR. COWELL: You did use the RGE peptide. Have you done an experiment where you tried to see if that inhibited an FHA negative but pertactin positive whole bugs?

DR. LEININGER: I have still not done any peptide inhibition with the whole bacteria yet.

DR. COWELL: I have one other comment. Mark Peppler brought up the point about cholesterol and I would caution about interaction of Tohama I or Tohama III to glycolipids. If you did the experiment with cholesterol, which we have done, Tohama I will also bind to cholesterol on a dot blot type of assay and it is specific because you can use cholesterol analogs and it does not bind. Tohama III does not. Tohama I mutants that are FHA negative will not bind to the cholesterol. So you can see these types of effects but there is no evidence that cholesterol is a receptor. I think you would have to be careful about interpreting such results.

DR. TUOMANEN: That was a really nice presentation but I am a little bit puzzled and have been for a while about the differences between the ability of pertactin to act as an adhesin for macrophages as you do it and then our inability to show the same. I am wondering, is it possible that the kinetics of these assays are different? In other words, as I understand from some of your published data, in your assay you allow adherence to take a very long course, and is it then possible that FHA might be the first step in adherence and pertactin may be something that adds on a perhaps stronger bond later in the course of the adherence interaction.

DR. LEININGER: In my cell attachment assay where the cells are attaching to purified protein, it is a one-hour assay. In the bacteria attachment assay, you are right, it is a longer four-hour assay. We have also looked at the kinetics with time and you brought up a very interesting point which we have been observing and wanting to speculate on. We would say that FHA would be the first adhesin of the bacterium and that later on, attachment will play a major role in adherence in maybe a tighter and closer binding of the bacteria to the cell surface.

Mechanisms of *Bordetella pertussis* Invasion of HeLa 229 Cells

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The interaction of *Bordetella pertussis* with human epithelial cells can involve attachment (15, 23) and internalization (4, 12). As with other invasive pathogens, the process of internalization into nonprofessional phagocytes is that of an induced phagocytosis, requiring the participation of living bacterial cells and host cell actin microfilaments (3, 7, 9). The present study assessed the effect of other inhibitors of microfilament formation on the invasiveness of *B. pertussis* and tested the role of the 69kDa outer membrane protein (pertactin) in the invasion process (E. Leininger, J. G. Kenimer, M. J. Brennan, these proceedings).

The formation of F actin in invasion can be inhibited by the fungal metabolites, the cytochalasins. By binding to actin, the cytochalasins inhibit actin polymerization (22), preventing the internalization of organisms within phagosomes. Because of this interaction, the cytochalasins are commonly used to assess the involvement of actin microfilaments in an invasive process. However, the cytochalasins also disrupt the external cell morphology of treated cells (1, 8, 13) which could affect their ability to interact with potentially invasive organisms. In contrast, the tumor promoter phorbol myristate acetate (PMA) has a profound effect on cytoskeletal protein distribution through its activation of protein kinase C (11, 17, 19, 21). Despite its effect on microfilament formation, PMA only mildly alters the external morphology of treated cells (17, 20). In this study, PMA, a functional analog of PMA (mezerein), and a nonfunctional analog of PMA (α4-phorbol-12, 13-didecanoate or 4PDD) were tested for their ability to inhibit invasion of HeLa 229 cells by *B. pertussis*. In particular, PMA and its analogs were compared with the cytochalasin B and D to see if their effects on cell morphology also influenced attachment of *B. pertussis*.

Within its mature form, pertactin has an Arginine-Glycine-Aspartic acid (RGD) motif (2) which is found on a number of proteins involved in cell attachment (6). Such proteins, like fibronectin, laminin, and vitronectin attach to a group of cellular receptors known collectively as integrins (18). That organisms can use integrins as receptors for invasion through an RGD sequence is illustrated by *Yersinia* sp. (10). Since *B. pertussis* contains RGD sequences in pertactin and filamentous hemagglutinin (FHA) (16), this study examined whether synthetic peptides constructed from these sequences would inhibit invasion of the organisms. In addition, monoclonal antibodies directed against pertactin RGD regions and FHA were tested for their ability to affect invasion.

MATERIALS AND METHODS

Bacteria and growth conditions. *B. pertussis* strain BP338, a naladixic acid-resistant derivative of Tohama I, obtained from Alison Weiss, Virginia Commonwealth University, Richmond, Va. was used in this study. Organisms were cultivated on Bordet-Gengou agar (Difco Laboratories, Detroit, Mich.) containing 15% defibrinated sheep blood. Cultures grown for 2 days were suspended to an optical density of 0.12 at 540 nm in Hanks balanced salt solution buffered with 25 mM of HEPES (*N*-2-hydroxyethylpiperazine-*N*'-ethanesulfonic acid, pH 7.2). Dilutions of organisms in Hanks-HEPES were then used for the invasion studies.

Invasion assay. HeLa 229 cells (human epithelium-like; ATCC CCL 2.1) were used as previously described (4, 5). Briefly, 24-well tissue culture trays were seeded with approximately 7 x 10^4 cells per well 18 h before an assay in Eagle minimal essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 3% fetal calf serum (Flow Laboratories,

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Inc., McLean, Va.) (MEM-3%FCS). Approximately 9 x 10^6 CFUs of *B. pertussis* in 0.4 ml were added to each well and incubated at 37°C in 5% CO_2 for 5 h under static conditions to allow bacteria to invade the cells. Monolayers were washed twice, then reincubated with 1.0 ml of MEM-3%FCS containing 100 µg of gentamicin (GIBCO) per ml for 2 h to kill the remaining extracellular bacteria. After 2 h, the monolayers were washed, harvested, and plated onto Bordet-Gengou agar for quantitation of viable intracellular CFUs.

Adherence Assay. The adherence assay was conducted essentially as described for the invasion assay except that the gentamicin treatment of the monolayers was eliminated. This assay resulted in cell-associated CFUs that represented both internalized and externally bound organisms.

Treatment of HeLa cell monolayers with various disrupters of microfilament formation. Phorbol myristate acetate, mezerein, 4PDD, and cytochalasins B and D were obtained from Sigma Chemical Co., St. Louis, Mo. Stock solutions were made by dissolving the reagents in dimethylsulfoxide (DMSO), except PMA, which was dissolved in 100% ethanol. The stock solutions were stored at -70°C, then warmed and diluted as needed in MEM-3%FCS and added to the monolayers 1 h before the addition of organisms. Controls consisted of DMSO or ethanol at the highest concentration used for the test compounds. The compounds were left in the wells for the duration of the invasion assay.

Inhibition of invasion by synthetic peptides. Peptides were synthesized which represented the RGD regions of pertactin, fibronectin, and FHA (Table 1). Peptide solutions were diluted in MEM-3%FCS and 400 µl were added to HeLa monolayers to give a final concentration of 1 or 10 µg/ml. The peptides were allowed to incubate with the monolayers at 37°C for 1 h before organisms were added. Because of the reversible nature of their binding, the peptides were kept in the wells after organisms were added. Invasion of the monolayers by the organisms was assessed as described for the invasion assay.

TABLE 1. Synthetic peptides used for inhibition of HeLa cell invasion by *B. pertussis*

Name (abbreviation)	Sequence
Pertactin 1 (PRN1-RGD)	**ATIRRGDALAGGAC**
Pertactin 2 (PRN2-RGD)	**AGYTRGDRGFTGDC**
Pertactin 3 (PRN3-RGE)	**ATIRRGEALAGGAC**
Filamentous hemagglutinin 1 (FHA1-RGD)	**VTVGRGDPHQGVLC**
Fibronectin RGD (Fn1 GRGDSP)	**GRGDSP**
Fibronectin RGE (Fn2 GRGESP)	**GRGESP**
Fibronectin RGD 14aa (FN3-RGD)	**AVTGRGDSPASSKC**

Effect of monoclonal antibodies on invasion. Monoclonal antibodies directed against pertactin (BPE3 and F6E5) or FHA (M08-X3C and MO8-X3E) were diluted in suspensions of BP338 in Hanks-HEPES to obtain final concentrations of 50 µg/ml. Organisms were incubated in the presence of antibody for 1 h at 37°C to allow binding to occur. After 1 h, bacteria were washed once, diluted appropriately, and then used in invasion assays as described above.

Staining of HeLa cells for F actin. Rhodamine-phalloidin (Molecular Probes, Inc., Junction City, Oreg.) was used to visualize F actin in HeLa cells treated with the various compounds that disrupt microfilament formation. HeLa monolayers grown on four-well slides (Nunc, Naperville, Ill.) were treated with dilutions of the compounds for 1 h at 37°C and then fixed with 3.7% formaldehyde for 10 min at room temperature. The cells were washed with phosphate-buffered saline (54 mM Na_2HPO_4, 13 mM KH_2PO_4, 73 mM NaCl) after which they were extracted with acetone at 4°C for 5 min and dried. The monolayers were then covered with a solution of 5 U/ml of rhodamine-phalloidin in phosphate-buffed saline and incubated at room temperature for 30 min. After washing with phosphate-buffed saline, the cells were mounted in 90% glycerol, and 10% phosphate-buffed saline, and observed using epifluorescence with a 545-nm excitation filter and 720-nm emission filter on a photomicroscope (Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N. J.).

RESULTS

Effect of PMA, mezerein, and cytochalasins on HeLa cells. The study first compared the effects of various microfilament disrupters on F-actin distribution and cell morphology to see (i) if the different disrupters caused different patterns of rhodamine-phalloidin staining in the treated HeLa cells and (ii) what effect these compounds had on HeLa cell integrity. Figure 1 illustrates these effects. Panel A is an untreated control, and the filaments of normally polymerized F actin can be readily seen as bright bands, due to their binding of

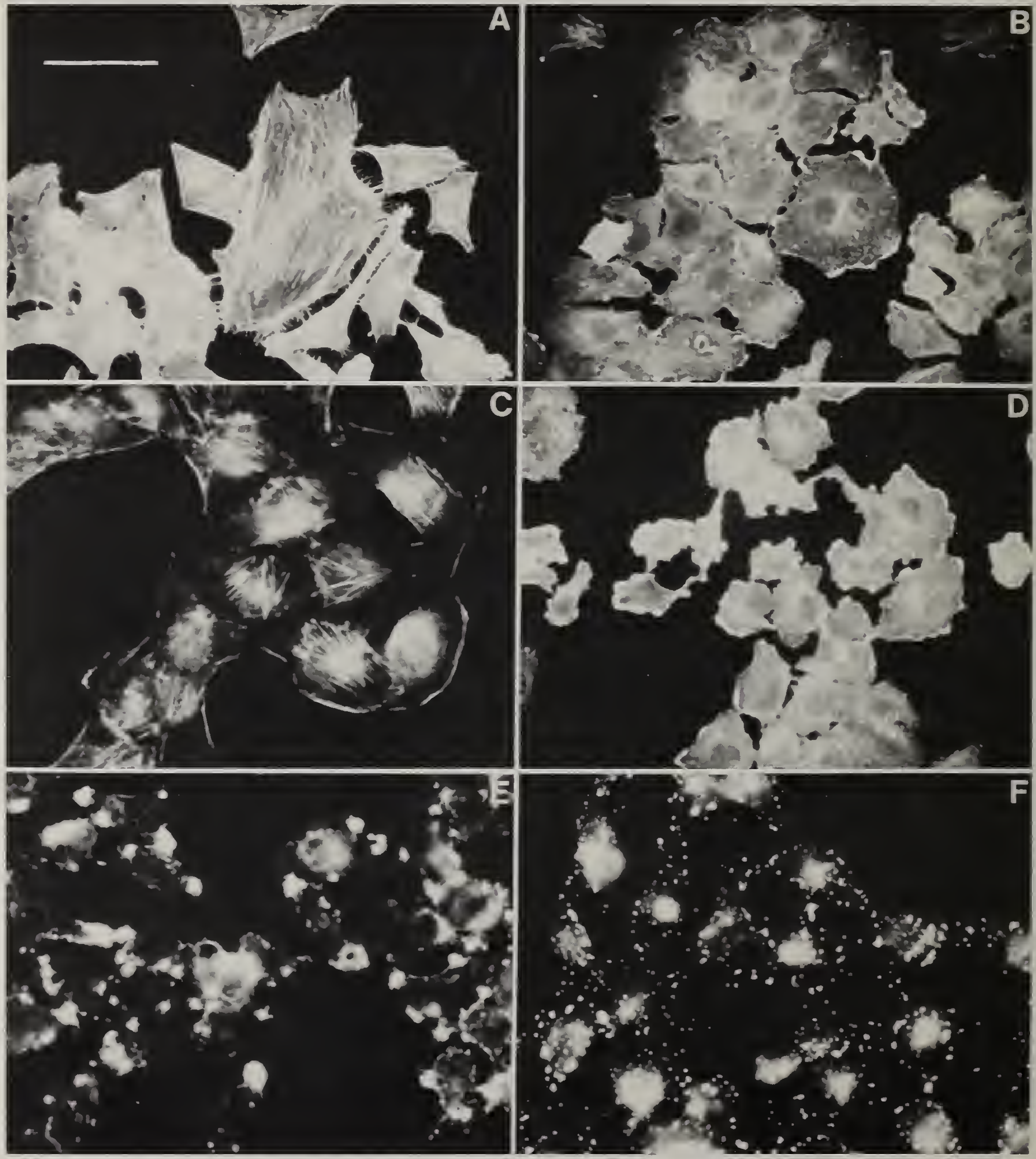

FIG. 1. HeLa cell monolayers stained with rhodamine-phalloidin and photographed with a fluorescence microscope to observe the effect of various disrupters of microfilament formation on F-actin patterns. The effect of the compounds on cell morphology can also be seen. All compounds were added to their respective monolayers and incubated 1 h at 37°C before processing. (A) Control with no additions. (B) 40 ng/ml of PMA. (C) 1,000 nM of 4PDD. (D) 100 nM of mezerein. (E) 2.5 μg/ml of cytochalasin B. (E) 2.5 μg/ml cytochalasin D. Bar = 5 μm.

rhodamine-phalloidin. Also note the shape of the normal HeLa cells, elongated and stellar in appearance.

Panel B of Fig. 1 shows a HeLa monolayer after treatment with 40 ng/ml of PMA for 1 h. In contrast to panel A, the brightly stained actin is located primarily around the periphery of the cells. The cells are somewhat less elongated than the untreated controls, but are still firmly adherent to the coverslip with no signs of blebbing. This effect could be seen down to 1 ng/ml of PMA.

Panel C is a HeLa monolayer treated with 1,000 nM of 4PDD, the inactive analog of PMA. As in panel A, the cells show normal filaments of F actin and normal cell morphology.

Monolayers of HeLa cells treated with 100 nM of mezerein, a functional analog of PMA (14), are shown in panel D. The HeLa cells in this sample look similar to those treated with PMA in panel B: i.e., actin staining in thin patches around the periphery of the cells, but an otherwise quite normal cell shape. This effect was seen at concentrations equal to or greater than 10 nM of mezerein.

In contrast to PMA and mezerein, treatment with the cytochalasins results in a more globular appearance of actin within the cytoplasm of the HeLa cells.

Panel E shows that cytochalasin B causes a larger, more aggregated staining, whereas cytochalasin D (panel

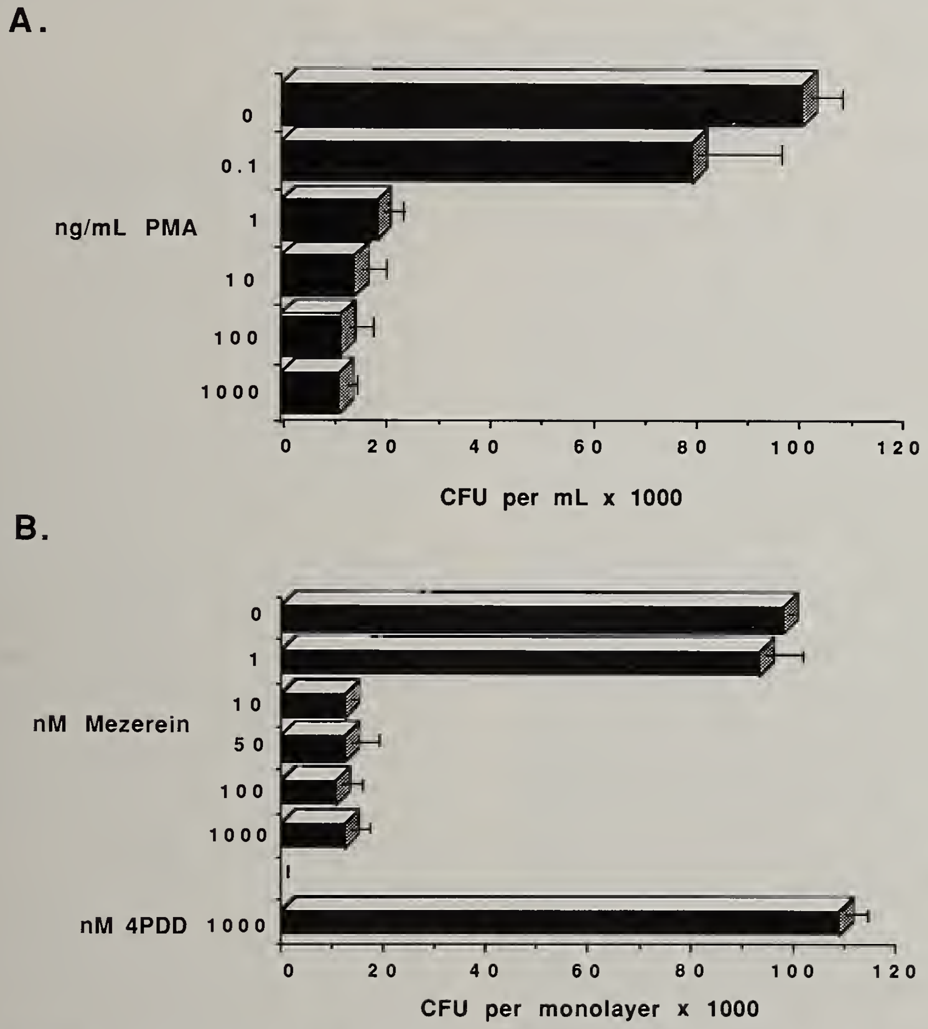

FIG. 2. Dosage thresholds for inhibition of HeLa cell invasion by *B. pertussis* strain BP338 with (A) PMA and (B) mezerein. (B) also shows that the inactive PMA analog, 4PDD, has no effect on invasion at the highest concentration tested (1,000 nM). The compounds were added 1 h before the organisms and left in the wells during the 4 h invasion assay. Values represent the mean ± SD of gentamicin-resistant CFUs from three independent experiments.

F) shows a more dispersed, almost spattered arrangement of stained material. For both of the cytochalasins, marked rounding and blebbing of the cells is seen and is most readily apparent in the monolayers before fixation and staining (data not shown).

Dose response of PMA and mezerein on invasion. From the data in Fig. 1, it was clear that PMA and mezerein had an effect on the formation of F actin. The study next focused on determining if the disruption of actin filaments seen in the HeLa cells also had a functional effect on internalization, i.e., the invasion of *B. pertussis* organisms. Figure 2A shows the dose-response effect of PMA on invasion of strain BP338. As little as 1 ng/ml of PMA inhibited invasion by approximately 80% of control values. At any of the concentrations used, PMA had no effect on the viability of the organisms. Mezerein showed a similar effect on invasion (Fig. 2B), although the minimum effective dose was greater (10nM). In contrast, the inactive analog 4PDD did not affect invasion even at 1,000 nM, the highest concentration tested.

Effect of various microfilament disrupters on adherence. It was suspected that the morphological changes in the HeLa cells caused by the cytochalasins (Fig. 1E and F), might affect the ability of the *B. pertussis* organisms to adhere. If true, this could cause misinterpretation of lowered cell-associated CFUs induced by these compounds as an inhibition of invasion, when, in fact, an inhibition of adherence was the real cause. Both cytochalasins have a significant effect on inhibiting adherence of *B. pertussis* to HeLa monolayers (Fig. 3). This is not the case with PMA or mezerein, even at the highest concentrations tested. No inhibition of adherence was observed with 1,000 nM of 4PDD.

Effect of synthetic peptides on *B. pertussis* invasion of HeLa cells. In addition to studying alterations of HeLa cell function on the interaction and invasion of *B. pertussis* organisms, the surface molecules on the bacteria that contribute to the invasion process were investigated. Likely candidates include molecules that possess RGD sequences that could possibly interact with integrin proteins on the surface of mammalian cells. The study attempted to determine if the specific RGD sequences from any of these molecules might interfere with invasion. Thus synthetic peptides were made based on the sequences of 2 molecules on the surface of *B. pertussis*, pertactin, and FHA. As controls, RGD peptides from the sequence of fibronectin were included. The RGD peptides were incubated with HeLa cell monolayers for 1 h prior to the addition of *B. pertussis* organisms (Fig. 4). Only the peptide based on the first RGD sequence in the pertactin open reading frame (PRN1-RGD) had a significant effect on invasion, lowering invasion to between 55 to 61% of controls. Neither the second pertactin RGD sequence (PRN2-RGD) nor an RGE analog of P1 (PRN3-RGE) had an effect on invasion of BP338. Similarly, the RGD sequences from FHA and the fibronectin peptides had no effect on invasion.

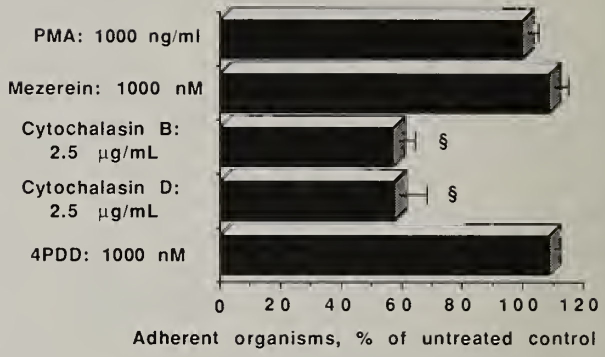

FIG. 3. The effect of various microfilament disrupters on the ability of *B. pertussis* strain BP338 to adhere to monolayers of HeLa 229 cells. The highest levels of PMA, mezerein, and 4PDD tested for invasion-inhibition were used, whereas the minimum invasion-inhibitory concentration of the cytochalasins were used. Values represent the mean ± SD of total cell-associated CFUs from three independent experiments. §, $P < 0.0005$, compared to untreated controls (student *t* test).

Effect of monoclonal antibodies to pertactin and FHA on the invasion of *B. pertussis*. Monoclonal antibodies were raised in mice against pertactin and FHA. The ability of these antibodies to inhibit invasion of *B. pertussis* was tested by incubating organisms in the presence of the antibodies for an hour at 37°C prior to conducting an invasion assay.

All the monoclonal antibodies tested, with the exception of MO8-X3E, significantly inhibited *B. pertussis* invasion of HeLa monolayers at a concentration of 50 µg/ml (Fig. 5). For the two preparations of monoclonal BPE3, this resulted in a reduction of invasion to approximately 61 to 75% of the control mean value; for F6E5, about 58% of control; and for MO8-X3C, about 78% of control.

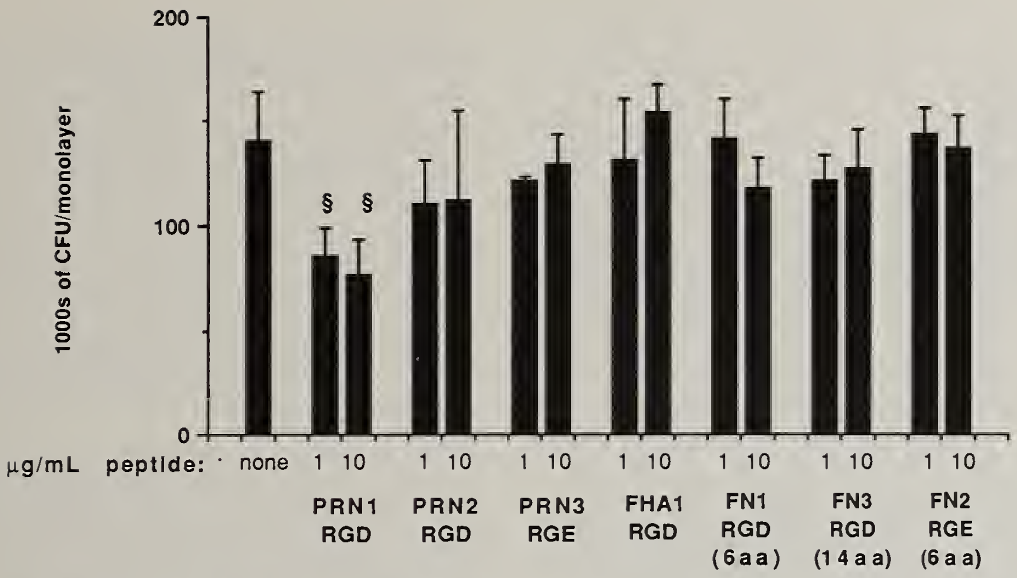

FIG. 4. The effect of synthetic peptides containing RGD sequences from pertactin (PRN), filamentous hemagglutinin (FHA), and fibronectin (FN), on the ability of *B. pertussis* strain BP338 to invade HeLa cells. Peptides were added to the HeLa monolayers 1 h before the organisms and left in the wells for the duration of the 4 h invasion assay. The identity and sequences of the peptides are listed in Table 1. The values plotted represent the mean ± SD for gentamicin-resistant CFUs from three independent experiments. §, $P < 0.0005$, compared to untreated controls (student *t* test).

DISCUSSION

Virulent *B. pertussis* can be internalized by human epithelial cells by a phagocytic, microfilament-dependent process (5, 12). Inhibition of microfilament formation can be a useful means of assessing the mechanism(s) involved in the internalization process, but other effects on the treated cells must also be taken into consideration to avoid misinterpreting results.

It has been shown that the cytochalasins have a noticeable effect on cell morphology (1, 8, 13). The data presented herein show that the cytochalasins affect HeLa cell morphology at the lowest concentrations that also inhibit microfilament formation. Importantly, at 2.5 µg/ml, the cytochalasins also significantly reduce adherence of *B. pertussis* to HeLa monolayers. Thus, the reduction of *B. pertussis* CFUs seen in invasion assays with the cytochalasins is at least in part due to an interference of bacterial binding to the epithelial cell surface, and not strictly a reduction in invasion as previously assumed.

As alternatives, the phorbol ester, PMA, and its nonphorbol analog, mezerein, both appear to alter microfilament formation without the concomitant loss of HeLa cell integrity. Moreover, these compounds can accomplish this alteration without interfering with the attachment of *B. pertussis* to HeLa cell monolayers (Fig. 3).

It is certainly likely that PMA and mezerein have other effects on the HeLa cells besides inhibition of microfilament formation, since they both affect protein kinase C. But neither compound lowered the viability of the HeLa cells, or of the *B. pertussis* organisms during the time span of these assays. Therefore, these compounds could prove useful in further understanding the pathways used by invasive organisms like *B. pertussis* to induce the host cell into engulfing them.

It is still not clear what the minimum antigenic requirements are for *B. pertussis* to invade mammalian cells successfully. It has been previously shown with Tn*5* or Tn*5lac* mutants of *B. pertussis* that specific loss of FHA or pertussis toxin results in significant reduction of HeLa cell invasion (5). The same study showed that other, unidentified *vir*-regulated genes resulted in lower levels of invasion; monoclonal antibody specific for lipooligosaccharide A, a non-*vir*-regulated molecule, also reduced invasion to approximately 42% of controls (5). The present study also tested the role of pertactin, and specifically the RGD portion of the molecule, as a potential mediator of *B. pertussis* invasion.

Data strongly suggest that the first RGD sequence of pertactin plays a role in invasion at some point during the process (Fig.4). It is worth emphasizing that none of the other RGD-containing peptides homologous to sequences from FHA or fibronectin had a significant effect

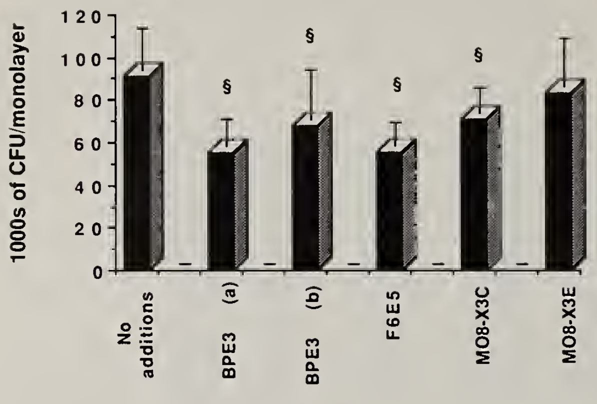

FIG. 5. The ability of monoclonal antibodies specific for pertactin (BPE3 a and b, F6E5), or FHA (MO8-X3C, MO8-X3E), to inhibit invasion of HeLa 229 monolayers by *B. pertussis* strain BP338. Antibodies were diluted to give a final concentration of 50 µg/ml and were preincubated with organisms for 1 h at 37°C. Organisms were washed once and added to monolayers to assess invasiveness. The plotted values represent the mean ± SD of gentamicin-resistant CFUs recovered for five independent experiments with the exception of antibody F6E5, where $n = 3$. §, $P < 0.0005$ (two-factor analysis of variance).

on invasion. Nor did the control PRN3-RGE pertactin peptide possessing the single amino acid substitution have a significant effect. Although the level of inhibition was between 55 and 61% of controls, these results nevertheless suggest that pertactin participates in invasion of host cells.

The monoclonal antibody data (Fig. 5), in general, support the findings of the peptide-inhibition experiments, again suggesting that pertactin contributes to HeLa cell invasion of *B. pertussis*. It was previously reported that monoclonal antibody BPE3 did not inhibit invasion (5). In the present study, it was shown that two additional preparations, a and b, are inhibitory. A third preparation, which had been lyophilized, showed no inhibition (data not shown). This inconsistency underscores the importance of appropriate purification, storage, and handling in the assessment of the biological functions of monoclonal antibodies. To further support the current results, an additional anti-pertactin monoclonal, F6E5 (whose epitope includes PRN-1; see I. G. Charles et al., this volume), also showed significant reduction of invasion (Fig. 5).

It is not surprising that the monoclonal to FHA, MOX-X3C, also inhibited invasion. It has been shown previously that polyclonal goat immunoglobulin G anti-FHA inhibits *B. pertussis* invasion of HeLa cell monolayers (5). As mentioned above, Tn*5* FHA$^-$ mutants also showed marked reduction in invasion (5). It is likely that anti-FHA is interrupting invasion by interfering with adherence (15, 16, 23), possibly using the same mechanism by which the monoclonal antibodies to pertactin inhibit invasion.

In their assay system, Lee et al. found FHA mutants to show levels of invasion similar to parental controls, thereby disputing a role for FHA in invasion (12). Future studies that assess the effect of combinations of pertactin and FHA monoclonal antibodies on both attachment and invasion in different cell systems may resolve some of these contradictions.

To date, the only *B. pertussis* mutants that appear to be virtually devoid of invasiveness are those with *vir* mutations affecting the expression of the majority of *vir*-regulated genes (12). All other single-gene mutants show only a partial reduction in invasion (5, 12). This finding suggests that invasion is a multi-factored event requiring several *vir*-regulated, and possibly non-*vir*-regulated, components (e.g., lipooligosaccharide A). Pertactin-minus and double mutants of pertactin and FHA would be useful for determining the combined contributions of these important proteins in attachment and invasion. Just how invasion is promoted by interaction of individual components, and in what sequence, will be fascinating to decipher.

ACKNOWLEDGMENTS

This work was supported in part by an Alberta Heritage Foundation for Medical Research studentship grant (to C.A.E.) and grants MA10415 from the Medical Research Council of Canada and ALA/89-080 from the Alberta Lung Association (to M.S.P.).

We thank San Vinh and Richard Sherburne for photographic assistance, Gordon Flowerdew for statistical analyses, and Judy Regan and Kenneth Seamon for the synthesis of the peptides used in this study.

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DISCUSSION

DR. HANSKI: I would like to ask you about the PMA effect. How was the exposure of the cells done with the PMA because longer exposure to PMA reduces the level of PKC in the cells.

DR. PEPPLER: This was a one-hour exposure to PMA initially and then through the invasion assay, PMA was maintained in the assay. During the time course of invasion and processing for measuring viable accounts, the PMA had no effect on viability of the organisms. I should have mentioned that it is pretty critical. Nor did it have an effect on the viability of the HeLa cells themselves based on trypan blue exclusion.

DR. SHAHIN: Your observation that a monoclonal to LOS inhibited invasion was quite striking. Could you speculate a little bit more on the mechanism by which you think your antibodies that inhibit may be working? Do you think you are just aggregating since some of these are agglutinating antibodies or in the case of the LOS, do you think it is blocking a determinant nearby that is really actually the one that is critical for the interaction?

DR. PEPPLER: The antibody was used in sub-agglutinating concentrations, so at least in the assay we were not agglutinating the organisms. Inhibiting the surface charge of *Bordetella pertussis* by covering up LOS A with a monoclonal might be one way that there is an effect on attachment and internalization. Alternatively, some of the things we talked about yesterday about exposure of other proteins mediated by the presence of LOS A, associated with outer membrane proteins might have bearing on that, too. At the moment we do not know.

DR. MASURE: I would like to comment just briefly on your speculation about *vir* regulation following internalization. We had some preliminary data where we created a mutant that constituatively expresses adenylate cyclase. In other words, it is no longer regulated by *vir*. We then looked at CR-3 mediated uptake into macrophages which is clearly different from your system and we then monitored the adenylate cyclase as it was internalized into the cell, and preliminary data suggest that indeed the adenylate cyclase is *vir* regulated. In other words, it is no longer expressed once it is internalized as compared to the constituative mutant which I will talk about tomorrow which always expresses the adenylate cyclase even after it is internalized in the macrophage.

DR. PEPPLER: That is really interesting. We were trying to use some of the vir-repressed gene mutants to look for alkaline phosphatase expression in cells and that turned out to be harder to do than we thought. Your report is encouraging.

DR. WEISS: I am really intrigued by the fact that you have been able to observe these in human patients. Can you talk about whether it took real heroic efforts to visualize this or whether it may be more common than we appreciated in the past.

DR. PEPPLER: The epithelial cells we got from humans but we grew those in vitro and infected them, so we have not yet actually gone into humans to look for intracellular organisms. That is a bit of a needle in a haystack but it certainly would be nice to be able to describe them there.

Speculating on what this might all be about, certainly organisms residing within cells are protected from antibody and complement and also the ravages of PMNs and so on, so it might be one adaptation to protect themselves against a normal immune system. The other is an intriguing one, too. It might be one way that the organisms are further carried and transmitted and this might be one of the ways of solving the enigma of carriage with *Bordetella pertussis* but again, that is easy to say and hard to prove.

DR. HEWLETT: You alluded to the issue of intracellular proliferation. Have you addressed that issue any further? Do you know whether they do proliferate and anything about the rate? You also mentioned that you do not see intracellular microcolony formation like occurs with other invasive organisms. Is there any possibility that they spread within a monolayer? Can you infect a monolayer with just a few cells that have organisms in them and show that they end up in other cells in that model?

DR. PEPPLER: We actually have not done that. We have been basically using the HeLa cells as just a medium, if you will, for looking at more of the initial events and trying to inhibit and identify the components that mediate internalization. I think the HeLa cell system that we use is really quite limited in that it is not even a polarized tissue culture system. I know the original paper by Fishel and Crawford in 1959 looked at the reported proliferation of the organisms in tissue culture but we have not really looked for further growth of the organisms.

DR. HEWLETT: Do you have any idea about the organisms coming out? Part of the speculation obviously is they need to emerge at some point in time.

DR. PEPPLER: That is intriguing. Again, it would just be speculation but I would imagine that another inflammatory event like a subsequent viral infection might be the trigger that allows the organisms to come out. But again, that is something that is theoretically testable in vitro but whether it is really relevant in vivo we do not know.

DR. HEWLETT: So as far as you can tell, there are no adverse effects on the infected cell.

DR. PEPPLER: That is what is intriguing about it, the organisms become, if you will, quiescent inside the HeLa cells, and if, in fact, they do modulate to *vir* minus, that might be the reasons why they neither cause damage to the cells once they get inside and also do not show additional proliferation from that point, at least not in the sense that we see for enteric invasion.

DR. BEMIS: Along the same lines, I may have missed it but there seems to be a fair bit of ambivalence regarding the use of the word internalization versus invasion. Do these organisms have to be metabolically active to get into the cells?

DR. PEPPLER: Yes, they do. That is a good point. That is why, at the outset, I said, virulent and viable organisms. Non-viable organisms cannot get in. That is an important point. The terms invasion or internalization; I know some people have clearer ideas of what those things mean. I think virulent *Bordetella* organisms have the ability to get inside these cells and it is for us to find out why they developed the ability to do so.

Invasion of HeLa 229, Chinese Hamster Ovary, and U937 Cells by *Bordetella pertussis*

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The causative agent of whooping cough, *Bordetella pertussis*, is a noninvasive pathogen which attaches to respiratory epithelial cells and produces disease through the action of several bacterial toxins. The virulence determinants involved in causing disease include filamentous hemagglutinin (FHA), several fimbriae, a 69-kDa outer membrane protein (OMP), pertussis toxin (PT), dermonecrotic toxin, adenylate cyclase-hemolysin toxin, and tracheal cytotoxin. The *vir* locus regulates expression of all of these virulence factors with the exception of tracheal cytotoxin (3, 22). The *vir* locus, which is also known as *bvg* (*Bordetella* virulence gene), has been sequenced and contains three open reading frames, *bvgA*, *bvgB*, and *bvgC*, which are predicted to encode proteins of 209, 175, and 936 amino acids, respectively (1). Phase variants that do not express the *vir*-activated factors contain a single cytosine inserted in a repeated sequence of cytosines in the *vir* gene (*bvgC*) resulting in a frameshift mutation (1, 18). The *vir* locus is also responsive to environmental factors such as nicotinic acid, magnesium and sulfate ions, and low temperatures (14, 19). When grown in the presence of these factors, virulent *B. pertussis* cells (X-mode cells) undergo reversible antigenic modulation to the avirulent C mode (11). Knapp et al. (9) have characterized a class of mutants called *mod* which constitutively express virulence factors in the presence of modulators and, as shown by DNA subcloning, map to the *vir* region.

Although the pathogenesis of whooping cough does not appear to require *B. pertussis* to invade host cells, the bacterium can survive inside macrophages (4) and it invades and survives in a nonprofessional phagocyte, HeLa 229 (7). Invasion of *B. pertussis* in HeLa 229 cells is mediated by microfilament-dependent endocytosis resulting in apparently nonreplicating bacteria surviving in membrane-bound phagosomes (7). Invasion requires *vir*-activated gene products. Consequently, the following did not invade: an insertion mutant in the *vir* locus (7, 12), four spontaneous Vir$^-$ phase variants, and bacteria that were environmentally modulated and thus did not express virulence determinants (12). However, mutations in several known and unknown *vir*-activated determinants did not abolish invasion to the level of Vir$^-$ strains (7, 12). None of 70 Tn*5*IS*50*$_L$::*phoA* (Tn*phoA*) insertion mutants were invasion-defective, suggesting that *B. pertussis* may possess redundant systems for invading HeLa 229 cells (12).

This report describes two unusual Vir$^-$ mutants and further defines the roles of FHA and 69-kDa OMP in invasion of HeLa 229 and Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

Bacterial strains. Table 1 describes the *B. pertussis* strains used in this study. These strains were grown on Bordet-Gengou agar (Difco Laboratories, Detroit, Mich.) supplemented with 1% glycerol and 20% defibrinated sheep blood (BG) or in Stainer-Scholte (SS) medium supplemented with cyclodextrin (8). Strains carrying the appropriate antibiotic markers (Table 1) were grown in media containing ampicillin (50 µg/ml), kanamycin (50 µg/ml), nalidixic acid (50 µg/ml), or tetracycline (5 µg/ml). Bacterial concentrations were estimated by optical densities at 550 nm. Viable bacterial concentrations in the inocula were confirmed for several experiments by titration on BG agar.

Host cells. All cell lines were purchased from American Type Culture Collection, Rockville, Md. HeLa 229

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* Presenting author.

TABLE 1. Bacterial strains and plasmids

Strain or Plasmid	Phenotype or genotype[a]	Reference or source[b]
B. pertussis		
Bp338	Nal^r mutant of Tohama I	23
Bp8001	Spontaneous nonhemolytic derivative of Bp338 selected on BG containing 0.25 μg of erythromycin per ml	12
Bp347	Bp338 *vir-1*: :Tn*5* Km^r Sm^r	23
Bp353	Bp338 *fha-1*: :Tn*5* Km^r Sm^r	23
Bp536	Sm^r mutant of Bp338	16
Bp101	*fhaB*Δ*101* in Bp536	16
Bp326	Spontaneous *vir* mutant of Tohama I, Sm^r Rif ^{r}; mutation due to cytosine insertion in *bvgC*	18
Bp18323	Mouse challenge strain for vaccine potency test	ATCC 9797
Bp9002	Spontaneous nonhemolytic derivative of Bp18323 selected on BG containing 0.1 μg of erythromycin per ml	12
SK16	Bp18323 *vag-16*: :Tn*phoA* FHA$^-$ Km^r Sm^r	9; Finn
SK75	Bp18323 *vag-75*: :Tn*phoA* FHA$^-$ Km^r Sm^r	9; Finn
SK91	Bp18323 *vag-91*: :Tn*phoA* FHA$^-$ Km^r Sm^r	9; Finn
SK301	Bp18323 *vag-34*: :Tn*phoA* *vir*: :pVI7-2 Ap^r Km^r Sm^r	Knapp
Bp1392-1	Hemolytic UV-exposed colony from clinical isolate Bp1392	6
Bp1394	Nal^r mutant of Bp1392-2, a UV-induced nonhemolytic derivative of clinical isolate Bp1392	6
Bp1397	Spontaneous nonhemolytic derivative of Bp1392-1 selected on BG containing 0.1 μg of erythromycin per ml	12
E. coli		
HB101	*FleuB6proA2recA13thi1ara14lacY1 galK2xyl5mtl1 rps*L20*sup*E44*hsd*20	2
Plasmids		
pGB304	*vir* insert in pLAFR2 IncP1 *cos* Tc^r	9
pVI7-2	0.93-kb *SalI-SalI* fragment of *bvgC* inserted in pJM703.1 *oriR6K mobRP4* Ap^r	9

[a] Nal^r, nalidixic acid resistant; KM, kanamycin; Sm, streptomycin; Rif, rifampin; Ap, ampicillin; Tc, tetracycline.

[b] Numbers refer to literature citations in text. Finn: Finn et al., unpublished data. Knapp: S. Knapp and J. J. Mekalanos, unpublished data.

cells were grown in a 5% CO_2 atmosphere in Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum. CHO cells were grown in a 10% CO_2 atmosphere in F12 medium supplemented with 10% fetal calf serum. U937 cells were grown in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal calf serum. Cell concentrations were determined by counting trypan blue-excluding cells in a hemacytometer. For a typical invasion assay, 25-cm^2 flasks were seeded with 10^6 cells 24 h before infection. U937 cells were seeded in medium containing 500 ng phorbol myristate acetate (Sigma Chemical Co., St. Louis, Mo.) per ml to promote cell spreading and other macrophage-like properties (15, 20).

Invasion assay. Nonconfluent monolayers containing 2 x 10^6 (HeLa 229 and CHO) or 5 x 10^5 (U937) cells were inoculated with 100 CFU of *B. pertussis* per cell in the appropriate tissue culture medium containing 2% fetal calf serum. The infected cells were incubated at 37°C and 5% CO_2 for the times specified and washed three times with phosphate-buffered saline containing $MgCl_2$ and $CaCl_2$. The extracellular *B. pertussis* cells were then killed by an antibiotic which does not penetrate host cells. Cells were incubated in 100 µg of polymyxin B sulfate (Sigma) per ml at 37°C and 5% CO_2 for 1 h. This procedure reduced the number of viable extracellular *B. pertussis* cells from 1 x 10^8 to 2 x 10^2 without injuring HeLa 229 cells as determined by trypan blue exclusion. The monolayers were then washed three times with phosphate-buffered saline containing $MgCl_2$ and $CaCl_2$. The cells were detached by gentle shaking with approximately 25 5-mm glass beads in 1 ml of phosphate-buffered saline. The entire cell suspension was then placed in a 25-ml Erlenmeyer flask containing approximately 45 glass beads, and the intracellular bacteria were released by sonication in a bath sonicator for 5 min at 8°C. The number of viable intracellular *B. pertussis* cells was determined by titration on BG agar.

Attachment assay. The researchers infected monolayers with *B. pertussis* as for invasion assay except that they placed 25-cm^2 flasks of cells on ice during the experiment to avoid invasion (12). At specified times, the monolayers were washed, the cells detached, and cell-associated bacteria enumerated as described for invasion assay.

Identification of *vir*-activated factors. Hemolysis was determined by unaided examination of *B. pertussis* colonies grown for at least 72 h on BG agar plates. Plates containing nonhemolytic colonies were further incubated for 24 h at 4°C to enhance any small hemolytic zones that may have been overlooked. The presence of FHA was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10) of extracts of *B. pertussis* cells grown on BG agar. The extracts were subjected to electrophoresis in a diallyltartardiamide-cross-linked 9% acrylamide gel followed by staining with Coomassie brilliant blue. Comigration with purified FHA (kindly provided by L. Winberry and R. Walker) identified full-length FHA of approximately 200 kDa. PT presence was determined in cell extracts and cell culture fluid by an enzyme-linked immunosorbent assay (ELISA) (13) in which microdilution plates were sensitized with 100 µg of fetuin (Spiro method, GIBCO Laboratories, Grand Island, N.Y.) per ml; PT bound to fetuin was detected with the monoclonal antibody Cx-4 (kindly provided by J. Kenimer), which is directed against the S1 subunit. Presence of the 69-kDa OMP in *B. pertussis* cell extracts was determined by Western blots (21) using the monoclonal antibody BPE3 (3).

RESULTS

***B. pertussis* invasion assay.** The assay used polymyxin B to inactivate extracellular bacteria. The sensitivity of *B. pertussis* to this antibiotic was inversely proportional to the age of the cultures. Thus, when 7 h, 24 h, and 48 h *B. pertussis* cultures grown in SS medium were treated with polymyxin B at normalized cell densities, the survival rates were 0.013%, 0.033%, and 0.17%, respectively. The researchers routinely used 24 h bacterial cultures in the invasion experiments; they used older cultures grown on BG agar in experiments with environmental modulators to monitor for hemolysis.

HeLa 229 monolayers infected with invasion-competent *B. pertussis* strains contained approximately 1 bacterium per cell, which is comparable to results obtained by Ewanowich et al. (7). This ratio was unaltered even when the multiplicity of infection was increased from 100 to 500 CFU per HeLa 229 cell.

Two unusual Vir^- mutants. The phenotype designation Vir^- describes strains which do not express known virulence determinants. The researchers determined that (i) a transposon mutant in *vir* (Bp347), (ii) several spontaneous phase variants including one strain in which the mutation was due to a C insertion in the C-repetitive sequence of *vir* (Bp326), and (iii) environmentally modulated Vir^+ strains did not invade HeLa 229 (Tables 2 and 3). They were subsequently surprised to discover two Vir^- mutants which retained their ability to invade HeLa

TABLE 2. Ability of *B. pertussis* phase variants, *vir* mutants, and *vir*-complemented strains to invade HeLa 229 cells

Strain	Hemolysin[a]	PT[a]	FHA[a]	69-kDa[a]	Percent invasion[b]
Bp18323	+	+	+	+	100[c]
Bp9002	–	–	–	–	0.22
Bp9002 (pGB304)	+	+	(+)	(+)	36
SK301	–	–	(+)	(+)	33
SK301 (pGB304)	+	+	+	ND[d]	ND
Bp1392-1	+	+	+	+	100[e]
Bp1397	–	–	–	–	1.3
Bp1394	–	–	(+)	(+)	120
Bp1394 (pGB304)	+	+	+	ND	ND

[a] Hemolysin, PT, FHA, and 69-kDa OMP were determined as described in the text.

[b] Bacterial strains were grown for 24 h in SS medium. Tetracycline was added to 5 µg/ml for strains containing the plasmid GB304. SK301 was grown in medium containing 50 µg of ampicillin per ml. Invasion was quantitated as the percentage of bacteria surviving polymyxin B treatment relative to the parent strains.

[c] 3.4 x 10^6 CFU.

[d] ND, Not done

[e] 2.4 x 10^6 CFU.

TABLE 3. Effect of environmental modulators on *B. pertussis* invasion of HeLa 229 cells

Strain	Modulators[a]	Hemolysin[b]	FHA[b]	Percent invasion[c]
Bp18323	–	+	+	100[d]
Bp18323	+	–	–	1.4
SK301	–	+	+	100[e]
SK301	+	–	–	0.7
Bp1392-1	–	+	+	100[f]
Bp1392	+	–	–	0.49
Bp1394	–	+	+	100[g]
Bp1394	+	–	–	5.7

[a] Bacterial strains were grown on BG agar at 35°C for 3 d (–), or BG agar supplemented with 5 mM nicotinic acid (Nic), 20 mM $MgSO_4$, for 4 days at 27°C (+). Bacterial cells were suspended in SS medium, and cell concentrations were determined by optical density at 550 nm. Nic and $MgSO_4$ were added to the tissue culture medium during the 5-h invasion when appropriate, although invasion experiments were done at 37°C.

[b] Hemolysin and FHA were determined as described in the text.

[c] Percentage of bacteria surviving polymyxin B treatment relative to the unmodulated cultures. Colonies were enumerated on BG agar without modulators.

[d] 6.6 x 10^5 CFU.

[e] 1.5 x 10^6 CFU.

[f] 7.5 x 10^5 CFU.

[g] 2.8 x 10^5 CFU.

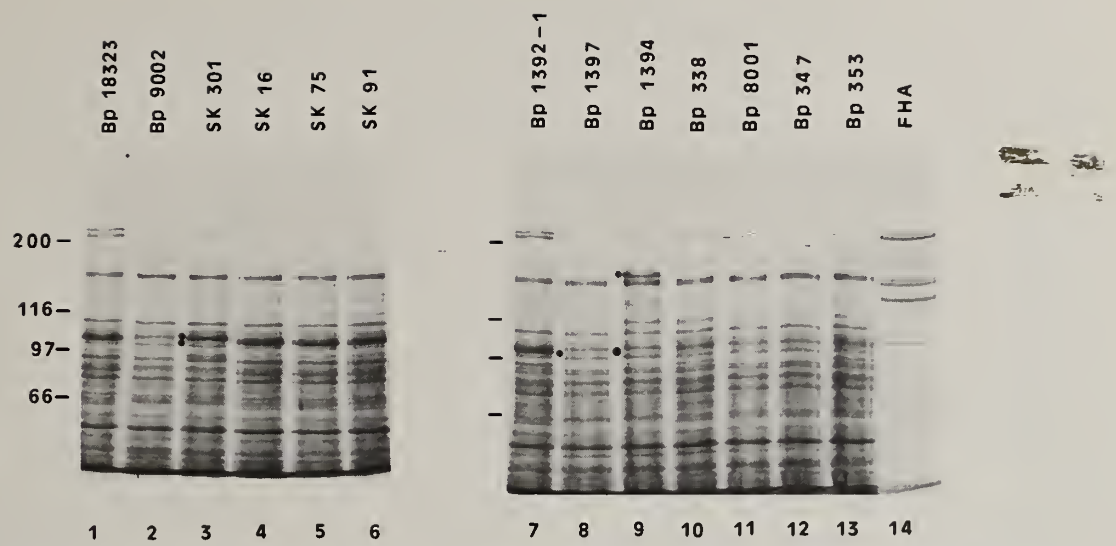

FIG. 1. SDS-PAGE analysis of proteins in *B. pertussis* cell. *B. pertussis* strains were grown in BG agar containing the appropriate antibiotics. The numbers on the left represent molecular mass standards in kDa. Lane 14 contains 4 μg of purified FHA. The arrow on the right denotes the two bands of approximately 200 kDa each interpreted as full-length FHA.

229 cells (Table 2). One of these (SK301) carried a plasmid insertion in the *vir* locus (S. Knapp and J. J. Mekalanos, unpublished data); the second (Bp1394) was a nonhemolytic colony which arose from a UV-irradiated population.

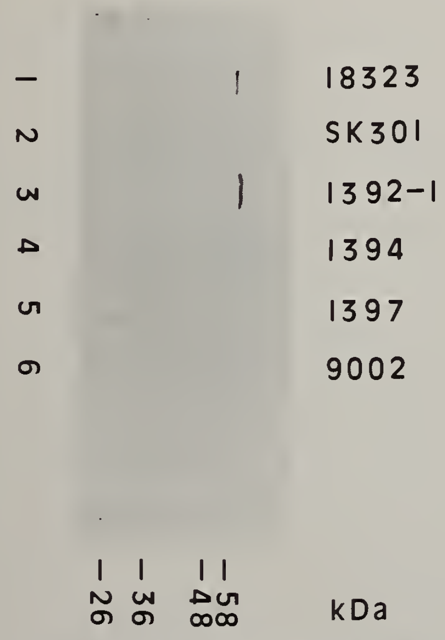

FIG. 2. Western blot analysis using the monoclonal antibody BPE3 (3) of *B. pertussis* cell extracts separated on an SDS-12% polyacrylamide gel. Equal amounts of protein were loaded in each lane as verified by staining with Coomassie brilliant blue (data not shown). The numbers at the bottom represent molecular mass standards in kDa. The arrow indicates the 69-kDa OMP.

The two strains had several characteristics in common with other Vir⁻ strains. For example, both were nonhemolytic and their PT (wild-type) levels were 0.3% (SK301) and 4.3% (Bp1394), as measured by an ELISA. These PT levels were similar to those obtained for spontaneous phase variants such as Bp9002. SDS-PAGE examination of their total protein revealed that both strains failed to express a 102-kDa protein (Fig. 1, lower circles in lanes 3 and 9); this protein was also absent in the spontaneous phase variants Bp1397 and Bp9002.

Unlike the other Vir⁻ mutants, however, SK301 and Bp1394 produced small amounts of full-length FHA (Fig. 1) and 69-kDa OMP (Fig. 2). Also, they each expressed at least one protein that is absent in the Vir⁺ parent strains and the spontaneous phase variants: a 170-kDa protein in Bp1394 (Fig. 1, upper circle in lane 9), and a 110-kDa protein in SK301 (Fig. 1, upper circle in lane 3).

SK301 and Bp1394 are indeed mutants in the *vir* locus because complementation with the *vir* plasmid pGB304 leads to production of hemolysin, PT, and FHA at levels equal to or approaching that of the Vir⁺ strains Bp1392-1 and Bp18323 (Table 2). However, the invasion-positive phenotypes exhibited by SK301 and Bp1394 are under *vir* gene regulation because they are sensitive to modulation signals (Table 3).

TABLE 4. Comparative uptake of *B. pertussis* by HeLa 229, CHO, and U937 cells

Strain[a]	*Vir* Phenotype	CFU		
		HeLa 229	CHO	U937
B. pertussis				
Bp338	+	1.0×10^6	3.1×10^6	3.3×10^5
Bp8001	–	6.0×10^3	ND	1.3×10^6
Bp347	–	6.0×10^3	1.6×10^4	2.0×10^6
Bp18323	+	1.7×10^6	ND[b]	2.3×10^5
Bp9002	–	7.0×10^3	ND	1.4×10^6
E. coli				
HB101		1.2×10^3	9.0×10^3	1.4×10^6

[a] *B. pertussis* and *E. coli* strains were grown for 24 h in SS medium and L broth (1% tryptone, 0.5% yeast extract, 1% NaCl), respectively.

[b] ND, not done

Invasion of CHO and U937 cells. The invasion of another nonprofessional phagocyte, CHO cells, was similar to invasion of HeLa 229 cells: the Vir$^+$ *B. pertussis* strains invaded, whereas Vir$^-$ strains and the noninvasive *Escherichia coli* strain HB101 did not invade (Table 4).

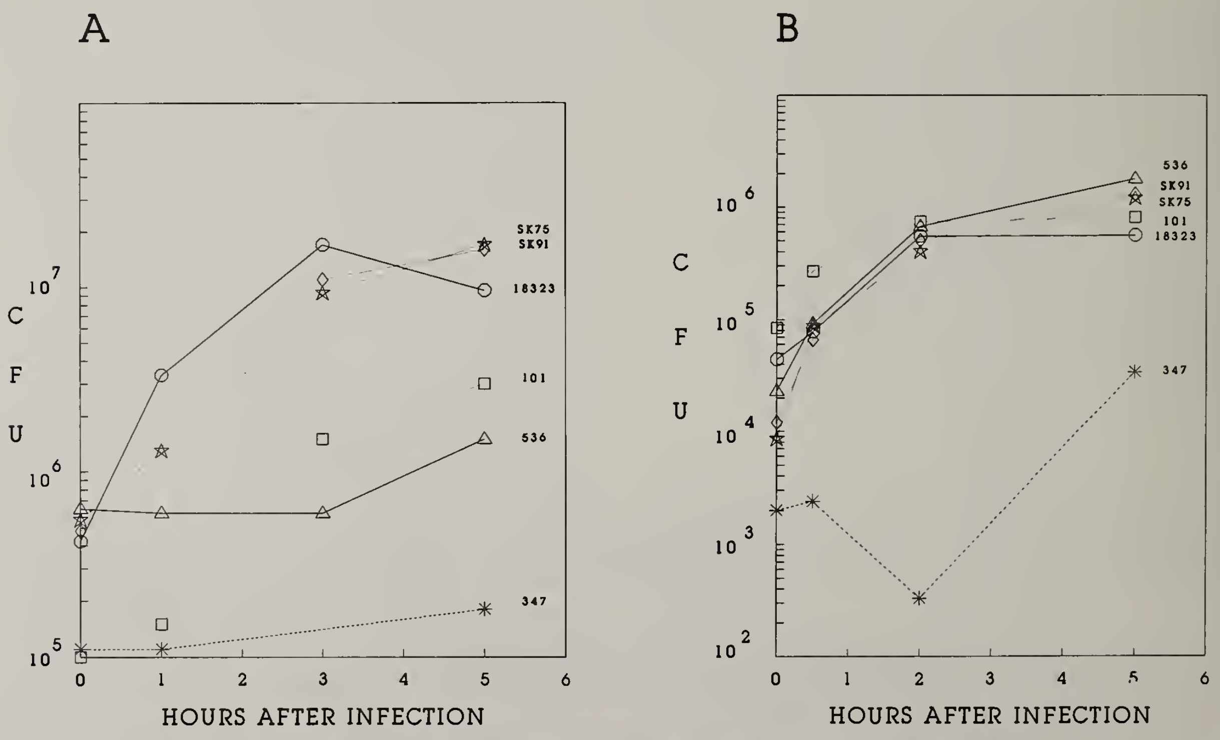

FIG. 3. Attachment (A) and invasion (B) of HeLa 229 cells. *B. pertussis* strains were Bp18323 (Vir$^+$) o, Bp536 (Vir$^+$) Δ, Bp101 (FHA$^-$) □, SK75 (FHA$^-$) ★, SK91 (FHA–) ◊, Bp347 (Vir$^-$)*.

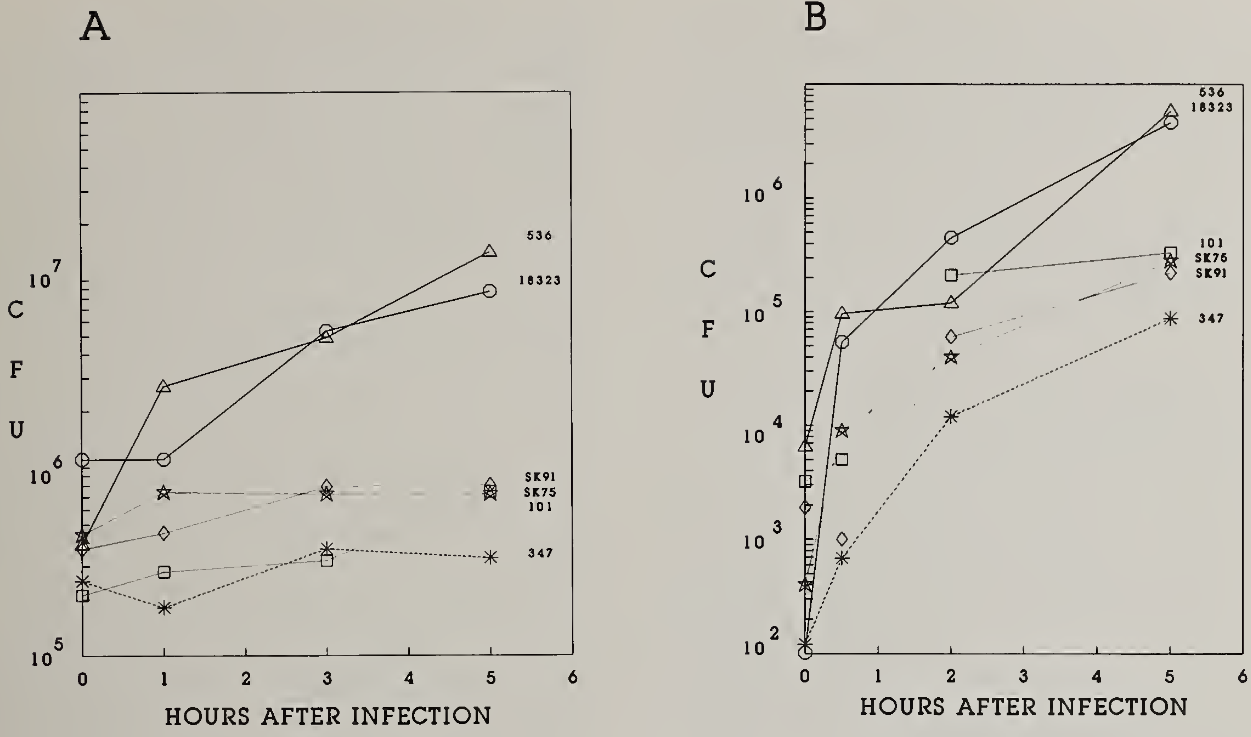

FIG. 4. Attachment (A) and invasion (B) of CHO cells. *B. pertussis* strains were Bp18323 (Vir$^+$) o, Bp536 (Vir$^+$) Δ, Bp101 (FHA$^-$) □, SK75 (FHA$^-$) ★, SK91 (FHA$^-$) ◊, Bp347 (Vir$^-$) *.

U937 is a cell line which assumes macrophage-like properties when treated with phorbol esters (20) and has been used in studies on *Legionella pneumophila* survival in macrophages (5, 15). In this study, both Vir$^+$ and Vir$^-$ *B. pertussis* strains and *E. coli* HB101 were taken up by U937 cells (Table 4).

Role of FHA in invasion. Although FHA is coregulated with invasion in strains SK301 and Bp1394 (Fig. 1, Table 2), the researchers found no evidence that FHA is involved in the invasion of HeLa 229 cells. The FHA mutants tested included the Tn*phoA* fusion strains SK75 and SK91 and a mutant which carries a deletion in the structural gene for FHA (Bp101) (16). SK75 does not produce any FHA. SK91, however, produces a small amount of FHA, as determined by an ELISA (T. Cabezon and C. Locht, personal communications); Bp101 does not produce full-length FHA, as determined by Western blot and hemagglutination of sheep erythrocytes (16). These three FHA$^-$ strains attached and invaded HeLa 229 cells to an extent similar to that of FHA$^+$ parent strains (Fig. 3A and 3B). Bp101's lower efficiency of attachment to HeLa 229 cells was not due to the FHA$^-$ phenotype because its FHA$^+$ parent (Bp536) also did not attach as well as the other Vir$^+$ strains.

Relman et al. (16) demonstrated a pronounced reduction in Bp101 attachment to CHO cells. The present research therefore used CHO cells to further investigate the role of FHA-mediated attachment in invasion by *B. pertussis*. Unlike the results obtained with HeLa 229 cells, the three FHA$^-$ mutants tested did not attach to or invade CHO cells as efficiently as the FHA$^+$ wild-type strains, although neither attachment nor invasion was reduced to the level of the *vir-1* mutant Bp347 (Figs. 4A and 4B).

DISCUSSION

Unlike the *vir* gene products expressed by spontaneous phase variants, those expressed by SK301 and Bp1394 were still capable of partially transactivating a subset of *vir*-activated genes. Strain SK301 is a plasmid insertion mutant in the *vir* gene of strain Bp18323. This strain was constructed by cloning the *SalI*-D fragment of *vir* (18) into the suicide vector pJM703.1 to yield plasmid pVI7.2, which was transferred into Bp18323 by conjugation. Because pVI7.2 cannot replicate in *B. pertussis*, ampicillin-resistant Bp18323 arose from the plasmid's integration into the *vir* gene by homologous recombination between the *SalI*-D sequences of *vir*. Thus, SK301 should be disrupted in sequences downstream of the *SalI*-D fragment. Examining the nucleotide sequence of the *vir* locus (1) shows that deleting sequences downstream of this fragment would truncate 85 amino acids from the C terminus of the bvgC polypeptide, resulting in an 851-amino acid polypeptide. This C-terminal sequence therefore may not be required for the positive regulation of invasion, although it is required for the regulation of other *vir*-activated genes. Because strain Bp326 did not invade, it is clear, however, that further truncation of bvgC eliminates expression of the invasion phenotype. This spontaneous Vir$^-$ phase variant carries a frameshift mutation in a repetitive cytosine sequence (18) which should result in the loss of an additional 49 residues from the C terminus of bvgC (resulting in an 802-amino acid polypeptide) compared with the mutation carried by SK301. Because Bp1394 was UV-irradiated, it is possible that the mutation which alters the *vir* locus in Bp1394 occurs at a site other than the repetitive cytosine sequence; it thus may have a phenotype similar to that of SK301.

The nucleotide sequence of the *vir* locus suggests that the *bvgA* and *bvgB* gene products may still be made in SK301 because these genes are located upstream of the *bvgC* gene in the operon. Roy et al. have shown that *bvgA*, when overproduced, is sufficient to activate the FHA promoter in *E. coli* (17). Given that SK301 and Bp1394 are the only two Vir$^-$ mutants which produce some FHA and 69-kDa OMP and are still invasive, bvgA also may directly activate promoters for the 69-kDa OMP and the putative invasion gene of *B. pertussis*.

The role of FHA and 69-kDa OMP in the invasion of HeLa 229 cells is implicated by the invasion-competent mutants Bp1394 and SK301. These strains differ from invasion-negative Vir$^-$ mutants in several proteins including the presence of FHA and 69-kDa OMP. In nonprofessional phagocytes, FHA's role in *B. pertussis* invasion appears to be dictated by whether FHA mediates bacterial attachment to the host cells. Attachment and invasion by FHA$^-$ mutants are reduced in CHO but not in HeLa 229 cells. Even in CHO cells, however, invasion by FHA$^-$ strains was not reduced to the level of Vir$^-$ strains, suggesting the existence of other *vir*-regulated attachment functions which are either partially redundant or more efficient at inducing uptake by HeLa 229 cells. The role of 69-kDa OMP needs to be further characterized using null mutations in the gene for this protein.

The present study assumed that the ability of *B. pertussis* cells to enter nonprofessional phagocytes such as HeLa 229 and CHO cells depends on bacteria-specified mechanisms regulated by the *vir* locus. These gene functions are not required for entry into professional phagocytes such as U937 cells, where entry is presumably mediated by host-specified mechanisms. The study's goal was to define the bacteria-encoded invasion functions. However, the low efficiency of invasion in HeLa 229 cells (1 bacterium per cell) and the lack of good evidence for intracellular replication led to the possibility that *B. pertussis* invasion of nonprofessional phagocytes is not due to bacteria-specified mechanisms but rather is the result of normal cell surface activities.

Vir$^+$ cells adhere to the host cell surface via proteins such as FHA and 69-kDa OMP (16; Li, Z. M., E. Leininger, A. Bhargava, S. Stibitz, I. G. Charles, N. F. Fairweather, M. Roberts, P. Novotny, C. R. Manclark, and M. J. Brennan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, B136 p. 49). Consequently, they are taken up to a higher extent than are Vir$^-$ cells. These *vir*-specified attachment factors are unimportant in uptake by professional phagocytes probably because the U937 cells efficiently engulf everything that comes in contact with them.

Consistent with the literature, this report uses the term "invasion" to describe the intracellular localization of *B. pertussis*; however, a more appropriate term is "uptake and intracellular survival." Even if *B. pertussis*'s entry into nonprofessional phagocytes is accidental, further investigation of its ability to persist intracellularly will determine if it is a potential mechanism for establishing asymptomatic carrier states in humans.

ACKNOWLEDGMENTS

These studies were supported in part by Public Health Service grant AI-26289, from the National Institute of Allergy and Infectious Diseases (to J. J. M.). We thank G. Siber for his continued support and encouragement. We are most grateful to A. Weiss, D. Relman, and S. Falkow for *B. pertussis* strains and to M. Brennan and J. Kenimer for monoclonal antibodies. Portions of this article were previously published (12).

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DISCUSSION

DR. HANSKI: Did you do electron microscopy and look where the *vir* minus and the *vir* plus are in the cell?

DR. LEE: We have not done that and we will probably have to look very closely in order to find that. As I was telling you, with the *vir* plus, we are only getting one bacterium per cell and for the *vir* minus, they are two logs lower so we have to examine probably 100 or more whole cells in order to find one intracellular bacterium.

DR. PARKER: Another possible explanation for the rising curve with the *vir* minus might be intracellular replication. That is to say, you have washed away the polymyxin, etc., and I think it might be important to rule out whether the cells are replicating intracellularly. One cannot really distinguish that for your *vir* minus as well.

DR. LEE: Right, that is something that we are very much interested in is whether the *vir* minus bacteria would be able to replicate intracellularly.

DR. FRIEDMAN: It was interesting that your data show that FHA does not seem to play a significant role in invasion of HeLa cells. Mark Peppler's work seemed to show the opposite. How do you --

DR. LEE: I do not know. We have discussed this. I really have not explanation. The assays are quite similar but that are different in a couple of areas, such as we use polymyxin B instead of gentamicin but I talked to Mark yesterday and I guess they have also tried polymyxin B and they are still finding that in their hands even with treatment with polymyxin B, the FHA minus mutants do not invade in HeLa 229 cells as well as *vir* plus. The other difference is the way we take the cells out. They use trypsin to take the cells out and we use glass beads.

DR. FRIEDMAN: The other question I had was about the studies you did in U-937 cells, the dramatic numbers of *vir* plus and *vir* minus organisms that were taken up. Have you looked over longer times at survival?

DR. LEE: No, that experiment is currently being done.

DR. GUPTA: In one of the figures you showed that at different intervals, starting at zero to one, two, three, four, five hours, at the zero hours, they started at 10^4 colony forming units. I just want to clarify that at zero time you got so much invasion when you just washed the cells with polymyxin B.

DR. LEE: For the attachment?

DR. GUPTA: No, just invasion.

DR. LEE: Is there a time that we wash with polymyxin B? We do.

DR. GUPTA: In the assay how can you get so much invasion at zero time when there is no time for the bacteria to go into these cells?

DR. LEE: I think the zero time numbers are similar to what we would get if we were just treating *Bordetella pertussis* with polymyxin B. It is not 100 percent kill. It is a six log drop-off.

DR. GUPTA: At zero time you start from the 10^4 colony forming units.

DR. LEE: Oh, you mean for the *vir* plus. We do not do the zero times here on ice and it is entirely possible that the polymyxin B is not killing immediately. It takes one hour to kill, and during that time perhaps some of them get inside. That is the only explanation that I can think of.

DR. RELMAN: Have you looked at the behavior of mod constitutive mutants or mutants that are unable to respond to environmental modulators in comparison with wild type to see whether there might be an effect on persistence or survival within the cells?

DR. LEE: Yes, we have looked at it just in terms of invasion after five hours and we did not see any difference but unfortunately the only mod mutants that we have are suppressed by one modulator or two. I think there is a mutant that can be suppressed by two modulators but any time you put in three modulators, then it could still be modulated. So I do not know what the intracellular environment is. That probably has more than two or three modulators.

DR. EWANOWICH: You stated, one bacterium per cell. That is assuming that each cell in the monolayer is equally infected. We find that that is not the case, and if we are lucky, maybe 50 percent of the monolayer cells are infected. If that were the case then you could not slice through the monolayer and look by EM and find more than one bacterium per cell. If you look back in the salmonella literature, that also states that if you are lucky, you will get 50 or 60 percent of the monolayer affected.

DR. LEE: Okay, thank you.

DR. WOLFF: Is it safe to use polymyxin? It is a polycation and has numerous effects on host cell membranes and internal metabolism of those cells.

DR. LEE: We use it because it seems to kill faster than gentamicin and sometimes we do do experiments with gentamicin just to confirm it.

DR. WOLFF: Because, for example, in an adrenal cell, you can stimulate steroidogenesis with polymyxin and other polycations so lots of things go on inside the cell and the difference in the total count between you and Peppler may be partly due to that.

DR. BROMBERG: Patients with symptomatic HIV disease could be infected with *Bordetella pertussis*, thus it was not a surprise when the first report of pertussis infection in a pediatrics AIDS patient was reported to us. Since then, there has been a report in an adult patient. Thus, respiratory tract secretions from any HIV patient should be examined for *Bordetella pertussis*.

In vivo work in the mouse pertussis model and the previously described in vivo work with human cells document the ability of *Bordetella pertussis* to invade pulmonary alveolar macrophages. Bronchial alveolar lavage (BAL) is used to diagnose pulmonary infections in AIDS patients. These BAL specimens are composed of alveolar fluid and large numbers of macrophages; thus BAL specimens would be an ideal place to look for

Bordetella pertussis. However, most patients with pulmonary pathology who have HIV infection, are given antibiotics before BAL specimens are obtained. This antibiotic therapy might make it impossible to culture *Bordetella pertussis*.

We were unable to culture *Bordetella pertussis* from 28 BAL specimens for HIV infected children. However, using indirect immunofluorescence microscopy with monoclonal BPG-10 directed against the lipooligosaccharide we found extracellular *Bordetella pertussis* in three of these 28 specimens.

In addition, all three specimens had *Bordetella pertussis* inside pulmonary alveolar macrophages. We found similar findings in an autopsy specimen in 1985 in a pediatric patient. Infection in a immunocompromised host may not represent unique pathogenic mechanisms, but rather exaggerations of normal mechanisms. Thus, we suggest an invasion of the pulmonary alveolar macrophage in vivo is involved in the pathogenesis of human *Bordetella pertussis* infection.

Structure-Function Studies of *Bordetella pertussis* Fimbriae

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Bordetella pertussis strains can produce two major fimbrial types, Fim 2 and Fim 3 (previously referred to as Agg 2 and Agg 3/6) (3, 18), which have subunit molecular weights of 22,500 and 22,000, respectively. The presence or absence of Fim 2 and Fim 3 determines the major serotype of *B. pertussis* strains. Like other bacterial fimbrial proteins, Fim 2 and 3 are effective immunogens which protect against infection by the parent bacterium. The murine protection induced by Fim 2 or Fim 3 against respiratory infection with *B. pertussis* shows some serospecificity (19). A mixture of Fim 2 and 3 is a major component of an acellular pertussis vaccine developed at the Centre for Applied Micro/biology and Research (CAMR). The vaccine has successfully completed phase-II trials in 3-month-old infants in the United Kingdom (E. Miller et al., this volume).

The aims of the present study were twofold. First, although the amino acid sequences of both Fim 2 and Fim 3 subunits are known and show considerable homology (13, 14), very little is known about their immunochemistry. Knowing how their immunogenicity depends on the secondary, tertiary, and quaternary structures of the subunits will aid in understanding and possibly optimizing the immune response to the fimbriae. Therefore we studied the effect of controlled denaturation/dissociation of fimbriae on their (i) physicochemical properties and (ii) reactivity with various monoclonal and polyclonal antibodies. These studies provide a basis for complete epitope analysis of the fimbrial subunits.

The second aim of this work was to assess the functional role of *B. pertussis* fimbriae during infection. By analogy with other gram-negative bacteria, the fimbriae would be expected to mediate adhesion of bacteria to mammalian cells. Only one study (6) has made this inference for *B. pertussis* fimbriae and filamentous hemagglutinin (FHA) appears to be the major adhesin in in vitro cell culture assays (23). Therefore we further examined the role of fimbriae in both the in vitro adhesion of *B. pertussis* to mammalian cells and the infection of laboratory animals.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. pertussis* strain Wellcome 28 (serotype 1, F2, 3) was used throughout for preparing fimbriae. The strain was maintained as freeze-dried suspensions and recovered by growing for 48 h at 35°C on charcoal agar containing 10% (vol/vol) defibrinated horse blood. The growth from plates was inoculated in 100 ml of CL medium containing 1 g/l of (2, 6-0-dimethyl) ß cyclodextrin (9) and incubated by shaking for 24 h at 35°C. From this primary culture, the researchers used 10 ml to inoculate 300 ml of CL medium in 2.5-l Thompson bottles; shaken incubation was then resumed for 48 h. Alternatively, the primary culture was used to inoculate 9 or 14 l of CL medium in an LH series 2000 fermentor. Growth was allowed for 35 to 40 h with pH controlled at < 8.2 and the medium's oxygen content maintained at 20% saturation. The bacteria were harvested by centrifugation (50,000 g, 30 min) and briefly washed in water.

Other bacterial strains were maintained either freeze-dried or frozen at -70°C and recovered by growth on charcoal agar plates. Strains B52, B86, and B7 were kindly donated by Dr. F. R. Mooi; these were derived from strain Tohama 536. Strains B52 and B86 (Fim 2^- and Fim 3^-) had a *sac* linker inserted in the *fim* 2 gene and a *kan* gene in the *fim* 3 gene. Strain B7 (FHA$^-$) was prepared by Dr. D. A. Relman and contained a *Bam*H1 deletion in the *fhaB* gene. Strains 1216 (PT$^-$), Tn99 (Vir$^-$), B862 (FHA$^-$), and B150 (FHA$^-$) were prepared by Tn*5* mutagenesis of strain Wellcome 28 by Dr. I. Livey and Miss M. Ward.

Preparation of fimbriae. Mixtures of Fim 2 and 3 were released from *B. pertussis* strain Wellcome 28 by cell homogenization; they were purified by repeated ammonium sulfate precipitation as described for Fim 2 (19). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to check the purity of the preparations (19). Both Fim 2 and Fim 3 bands were detected in all preparations, and the ratio of Fim 2 to Fim 3 was in the range 60:40 to 75:25. The researchers obtained 50 to 100 mg of fimbriae from a 10-l culture.

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* Presenting author.

Denaturation and renaturation of Fim 2 and 3. All experiments were performed with Fim 2 and 3 preparations denatured in guanidine hydrochloride (Gnd HCl) (Sigma UK). The extent of denaturation was monitored by fluorescence intensity and circular dichroism spectroscopy, reaction with various antibody preparations, and gel filtration.

(i) **Fluorescence.** Fim 2 and 3 in either phosphate-buffered saline (PBS), pH 7.2, or 0.125-M ethanolamine - 0.1 M NaCl, pH 10.5 (ethanolamine buffer) were diluted to constant protein concentration with varying Gnd HCl concentration by adding the appropriate buffer containing 6.5-M Gnd HCl. After incubation at 4°C overnight, aliquots were analyzed at 25°C in a Perkin-Elmer LS-5 fluorimeter using 1-cm quartz suprasil cuvettes (Hellma, UK). The excitation wavelength was 280 nm, and the emission from tyrosine residues was detected at 306 nm with slit widths of 2.5 nm and 5 nm for excitation and emission, respectively.

(ii) **Enzyme-linked immunosorbent assay (ELISA).** The ability of denatured and native fimbriae, prepared as above for fluorescence, to react with various antibody preparations was determined by ELISA as described previously (20), except that Fim 2 and 3 mixture was coated onto ELISA plates (Nunc immuno plates 1) at 2 µg/ml in the buffer used for denaturation. Monoclonal antibodies 2A, 51/24 (to Fim 2), and 3A (to Fim 3) were used as diluted cell supernatants (2). The murine polyclonal sera to peptides 17 and 18a, derived from the Fim 2 amino acid sequence, were prepared as described previously (3). Antiserum against SDS-denatured Fim 2 and 3 was prepared by immunizing mice with 10 µg of Fim 2 and 3, which had been treated with 1% (wt/vol) SDS and 1% (vol/vol) ß-mercaptoethanol at 100°C for 5 min in 25% (vol/vol) alhydrogel. After 6 weeks, mice were boosted with the same dose of antigen; 2 weeks later, they were bled.

(iii) **Gel filtration.** For these experiments, we transferred Fim 2 and 3 to an ethanolamine buffer, pH 10.5, containing various concentrations of Gnd HCl by a buffer exchange on Sephadex G25 PD-10 or NAP-5 columns (Pharmacia Uppsala, Sweden). The protein was then incubated at 4°C overnight and subsequently loaded onto a Superose 6 column (FPLC HR 16/50 Pharmacia) which had been equilibrated with the denaturation buffer. The column was eluted with appropriate buffer at a flow rate of 0.1 ml/min. Fractions (2 ml) were analyzed for fluorescence and antibody binding by ELISA.

(iv) **Circular dichroism (CD).** CD spectra were measured at room temperature in a Jasco J600 spectropolarimeter using 1-cm path length cells. Gnd HCl was added in increments to 3 ml of Fim 2 and 3 (500 µg/ml) in ethanolamine buffer, pH 10.5, and spectra were measured after 1 h. Six repetitive spectra were scanned for each solution and were computer-averaged. Computer manipulation deducted a similarly averaged solvent spectrum from the solute spectrum. The concentration of Gnd HCl in the solutions was calculated from refractive index measurements.

(v) **Renaturation.** Attempts to renature/reassociate denatured Fim 2 and 3 were made by sequential dialysis of 1 to 2 ml of Fim 2 and 3 (50 µg/ml) against 1-1 volumes of various buffers at 4°C or room temperature.

Preparation of FHA and pertussis toxin (PT). FHA and PT were prepared from culture supernatants of *B. pertussis* strain Wellcome 28 by hydroxylapatite chromatography followed by affinity chromatography on fetuin-Sepharose for PT (1) or gel-exclusion chromatography for FHA (7).

Intranasal infection of mice. Bacterial strains for infection of mice were scraped off charcoal agar plates after 24 h growth, dispersed in 1% (wt/vol) Casamino Acids and adjusted to the appropriate concentration (usually 2 x 10^7 organisms/ml). The viable counts of the inocula were subsequently determined by plating out appropriate dilutions. Mice of 5 to 7 weeks old were lightly anesthetized with diethylether. Next, a 50-µl bacterial suspension was placed on the tips of their noses for inhalation. In some experiments, mice were immunized with specific antigens by intraperitoneal injection of 0.5-ml antigen in 25% alhydrogel 3 weeks prior to infection. Infant mice of 5 to 7 days old were similarly infected with a 10-µl bacterial suspension; they were not given anesthesia, however.

At the appropriate time after infection, mice were sacrificed by either cervical dislocation (older mice) or by placement in a CO_2 atmosphere (infant mice). Colonization of the nasal cavity was assessed by sagital section of the skull and removal of tissue from the nasal cavity with a sterile toothpick washed in 1% (wt/vol) Casamino Acids to determine viable counts. Mouse lungs and trachea were carefully removed from the tho-

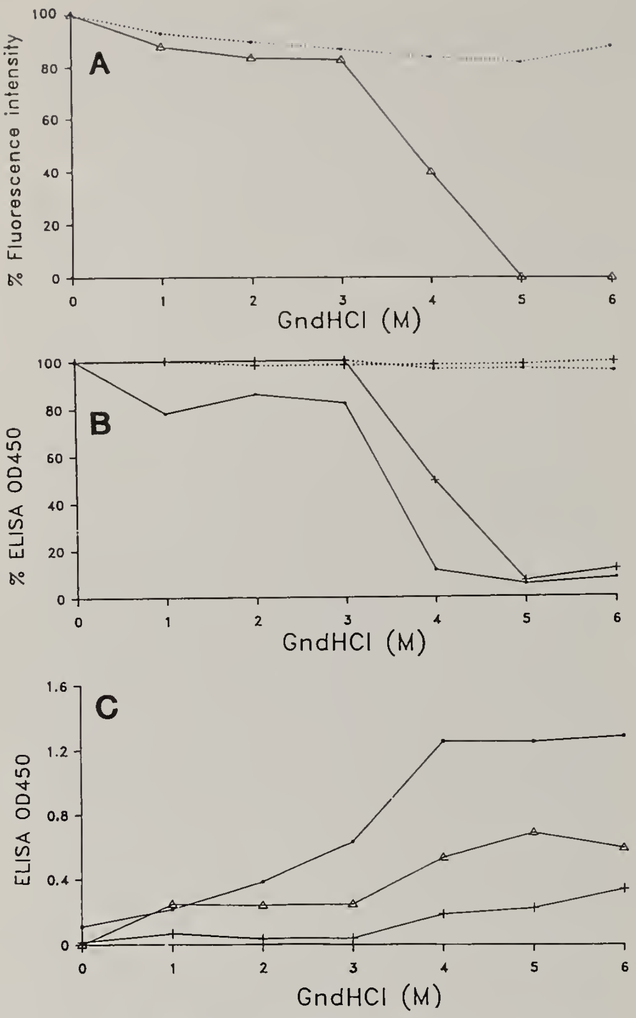

FIG. 1. Denaturation of Fim 2 and 3 with Gnd HCl. The fluorescence and antigenicity of suspensions of Fim 2 and 3 (39.2 µg/ml) in PBS, pH 7.2 (---) or ethanalamine buffer, pH 10.5 (—), containing various concentrations of Gnd HCl were determined. (A) Fluorescence intensity. Results are expressed as percentage fluorescence intensity of native fimbriae. (B) ELISA with monoclonal antibodies AGG 2A (+) and Agg 3A (·) at 1/800 dilution. Results are expressed as percentage OD_{450} of native fimbriae. (C) ELISA with polyclonal antisera raised against SDS-denatured Fim 2 and 3 at 1/30,000 dilution (·), peptide 18A at 1/10,000 dilution (Δ), and peptide 17 at 1/2,000 dilution (+). Results are expressed as OD_{450}.

rax after ventral section and removal of the rib cage, heart, and thymus. The lungs and trachea were then separated and placed in 10 ml 1% (wt/vol) Casamino Acids. Viable counts were made after the organs were homogenized for 10 sec in a Silverson homogenizer and appropriately diluted.

Vero cell adhesion assay. The assay was carried out essentially as described previously (6, 8). Vero cells (Green monkey kidney cells) were obtained from ECACC, CAMR, and grown on Eagle minimal essential medium buffered with HEPES (N-2-hydroxyethyl-piperazine-N^1-2-ethanesulforic acid) and bicarbonate and containing 10% (vol/vol) fetal calf serum (growth medium). We added 420 µl of a suspension of Vero cells, adjusted to 10^5 cells/ml, to the 24 wells of a tissue culture plate, each of which contained a sterile 13-mm-diameter circular glass cover slip. After incubation at 37°C for 18 h, the medium was removed and replaced by 420 µl of a suspension of 10^8 bacteria/ml in growth medium. After 2 h incubation at 37°C, the glass cover slips were washed three times in growth medium, fixed in ethanol, stained with 1% (wt/vol) crystal violet, and mounted on slides with De Pex. Dark field illumination was used to count the numbers of bacteria adhering to the edges of a representative number of Vero cells. Groups of data were analyzed for differences using nonparametric statistics.

The basic adhesion assay was modified in two ways to examine the specificity of adhesion. First, to assess antigens' ability to block adhesion, Vero cells were incubated with purified bacterial antigens for 1 h at 37°C and then washed three times in growth medium prior to infection with bacteria. Second, to assess the ability of antibodies to prevent adhesion, bacteria were pre-incubated with 10% (vol/vol) specific mouse serum for 30 min at 37° prior to infection of Vero cells. The controls for these two modifications were Vero cells or bacteria incubated in growth medium.

RESULTS

Dissociation of Fim 2 and 3. Eshdat et al. (5) and Karch et al. (10) in studies involving treatment of intact *Escherichia coli* fimbriae with high concentrations of Gnd HCl have provided useful data on the denaturation/dissociation of fimbriae and the immunogenic properties of the native and denatured forms. The experiments described below used a mixture of Fim 2 and 3, since sufficient amounts of separately purified Fim 2 and Fim 3 were not available.

The dissociation/denaturation of Fim 2 and 3 by Gnd HCl is very dependent on pH. No dissociation was detected at pH 7.2 with concentrations of Gnd HCl up to 6 M. At pH 10.5, however, a two-stage dissociation/denaturation of Fim 2 and 3 with increasing Gnd HCl concentration was observed; at Gnd HCl concentrations from 0 to 1 M, the intensity of fluorescence emission was slightly reduced; intensity was completely lost at between 3- and 5-M Gnd HCl (Fig. 1A). Reactivity with monoclonal antibodies to either native Fim 2 or Fim 3 showed a similar reduction with increasing Gnd HCl concentration; however, the transition at 0- to 1-M Gnd HCl was not observed with all antibodies (Fig. 1B). In contrast, the reaction of fimbriae with antiserum raised to SDS-denatured fimbriae or to synthetic peptides derived from the Fim 2 amino acid sequence increased with increasing Gnd HCl concentration (Fig. 1C).

Kinetic analysis of the fluorescence of Fim 2 and 3 in 3- to 5-M Gnd HCl indicated that structural transition was rapid and was completed in 120 min (data not shown); more detailed analysis of this fluorescence change showed a smooth transition (Fig. 2). In contrast, preliminary data on the minor transition occurring between 0- to 1-M Gnd HCl indicated a stepped transition which may correspond to separate structural changes in the Fim 2 and Fim 3 proteins (data not shown).

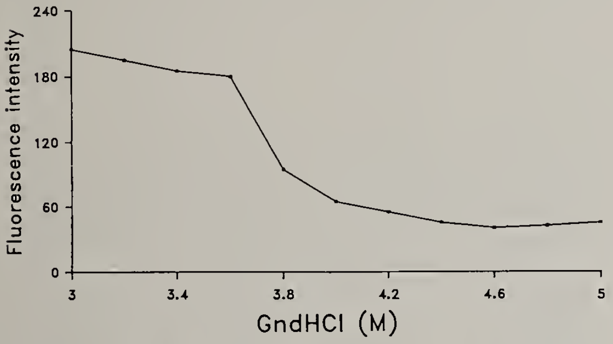

FIG. 2. Effect of 3 to 5 M Gnd HCl on the fluorescence intensity of Fim 2 and 3 in ethanolamine buffer, pH 10.5. Experimental details as for Fig. 1.

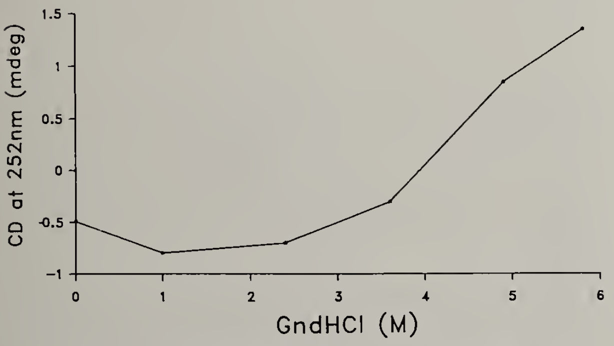

FIG. 3. Circular dichroism at 252 nm of Fim 2 and 3 in ethanolamine buffer, pH 10.5, containing 0 to 6M Gnd HCl.

The CD spectra of Fim 2 and 3 at pH 10.5 showed a weak negative Cotton effect with a minimum at 250 nm, the intensity of which was unchanged up to Gnd HCl concentrations of 3.6 M. At higher concentrations, this effect was abolished and replaced by a relatively strong positive peak with a maximum at 252 nm. The change in CD intensity at 252 nm with increasing Gnd HCl concentration (Fig. 3) showed that the transition occurred between 3.6-M and 5.8-M Gnd HCl. This finding agrees with results from fluorescence and ELISA measurements. In strong Gnd HCl, the Cotton effect with a maximum at 252 nm arises from ionized tyrosines (4). Thus, the transition observed by CD spectroscopy is probably due to alterations in tyrosine exposure to the solvent resulting from changes in the secondary or tertiary structure of the fimbriae.

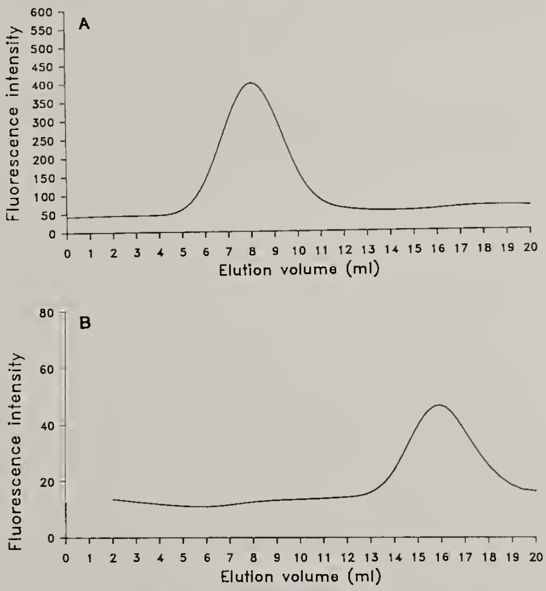

FIG. 4. Gel filtration on Superose 6 of Fim 2 and 3 in ethanolamine buffer, pH 10.5, containing (A) 1-M or (B) 5-M Gnd HCl. Fim 2 and 3 were equilibrated overnight at 4°C in either 1-M or 5-M Gnd HCl, then 200 to 250 µg run on a Superose 6 (HR 16/50 FPLC) column and eluted with appropriate buffer. Fluorescence measurements of fractions were made with excitation at 280 nm and emission at 306 nm.

The structural transition in Gnd HCl was studied by gel exclusion on FPLC (Fig. 4). In 0- to 1-M Gnd HCl Fim 2 and 3 eluted as a high molecular weight component in the void volume of the column; there did, however, appear to be a slight retention when the buffer contained 1-M Gnd HCl. In 5-M Gnd HCl, the fimbriae eluted as a major peak just after chymotrypsinogen (molecular weight 25,000), indicating complete dissociation into

TABLE 1. Antibody recognition of denatured Fim 2 and 3 following dialysis in potentially renaturing buffer

Antiserum	% maximum antibody binding activity after dialysis against[a]			
	5-M Gnd HCl pH 10.5	5-M Gnd HCl pH 7.2	1-M Gnd HCl pH 7.2	0-M Gnd HCl pH 7.2
Anti-Fim 2 and 3 (SDS[b])	100	62	27	32
Antipeptide 17[b]	100	9	5	5
Antipeptide 18A[b]	100	84	1	2.8
McAb 51/24[c]	0	0.5	0.5	1.0

[a] Fim 2 and 3 in 0.125 M ethanolamine, pH 10.5, containing 5 M Gnd HCl was sequentially dialysed against PBS, pH 7.2, containing 5 M Gnd HCl, 1 M Gnd HCl and 0 M Gnd HCl. Aliquots were removed at each stage for ELISA. The results are expressed as a percentage of maximum antibody binding.

[b] Antibodies raised against denatured fimbrial or peptides.

[c] Monoclonal antibody to Fim 2.

TABLE 2. Effect of preincubation of Vero Cells with antigens on the adhesion of *B. Pertussis*

Antigen	(µg/ml)	No. bacteria adhering per Vero cell[a]	
		Wellcome 28	Tn99
None		5	0
FHA	(10)	9[b]	0
PT	(23)	4	0.2
Fim 2 and 3	(21)	5	0

[a] Median number of bacteria counted in a random sample of 15 Vero cells.

[b] Significantly different to the number of bacteria adhering to Vero Cells which were not preincubated with antigen.

monomers (molecular weight between 22,500 and 22,000). This finding agrees with that of previous studies using sedimentation analysis (3). Several minor peaks were also observed with smaller apparent molecular weights. The dissociation of fimbriae into monomers at 5-M Gnd HCl was confirmed by laser light scattering studies of fimbriae in 0- to 5-M Gnd HCl (data not shown).

Renaturation/reassociation of fimbriae. Attempts to renature/reassociate fimbriae from Gnd HCl solutions were largely unsuccessful, although in some cases unstructured aggregates were observed in the electron microscope. Renaturation by sequential dialyses to change the pH of denatured fimbriae from 10.5 to 7.2, followed by reduction in the Gnd HCl concentration, was investigated by assessing the binding of fimbriae to antibodies in ELISA. The "renatured" fimbriae lost the ability to bind antisera to synthetic peptides and to denatured fimbriae. Although the loss indicated that some refolding had occurred, binding to monoclonal antibodies which interact with native fimbriae was, at best, only weakly restored (Table 1).

Involvement of fimbriae in attachment of *B. pertussis* to Vero cells. We studied the role of fimbriae and other antigens in the attachment of *B. pertussis* to Vero cells by examining (i) the adherence of bacterial mutants deficient in specific antigens, (ii) the ability of purified antigens to block receptor sites on Vero cells, and (iii) the ability of specific antisera to neutralize the bacterial adherence.

Comparing the Vero adhesion abilities of wild-type and various transposon mutants of *B. pertussis* showed that mutants unable to produce FHA (Bp862, Bp150) or all virulence components (Tn99) had a markedly reduced ability to adhere. In contrast, a strain which was deficient in fimbriae (Bp86) adhered in high numbers to Vero cells, perhaps even with enhanced adherence when compared to a wild-type strain (data not shown).

Preincubation of Vero cells with purified PT, FHA, or Fim 2 and 3 did not significantly reduce the adhesion of *B. pertussis* to Vero cells. In fact, FHA caused an en-

TABLE 3. Comparison of the ability of antigens to protect mice against lung infection and to induce antiserum preventing adhesion to Vero cells

Antigen	Dose per/mouse (μg)	Number of bacteria recovered from lungs (% control)	Number of bacteria adhering to Vero cells (% control)
PBS (control)	--	100	100
PT	10	0.1	20.4
PT	2	0.5	49.0
PT	0.4	0.7	57.1
FHA	10	0.5	20.4
FHA	2	27.0	22.4
FHA	0.4	20.0	73.5
Fim 2 and 3	10	1.5	6.1
Fim 2 and 3	2	4.5	38.8
Fim 2 and 3	0.4	28.1	42.8

Mice were immunized with the stated dose of antigen. Three weeks later, they were either challenged intranasally with *B. pertussis* and bacteria isolated from the lungs after another 7 days, or bled and the serum's ability to prevent adhesion to Vero cells determined.

hanced adherence of the virulent strain but was, surprisingly, unable to increase the adhesion of the Tn99 Vir$^-$ mutant (Table 2).

These observations suggested that FHA was the major adhesin of *B. pertussis* to Vero cells and that fimbriae were not involved in the adhesion. Previous CAMR studies had shown that monoclonal antibodies could prevent the adhesion of *B. pertussis* to Vero cells; the researchers decided to repeat these studies using polyclonal sera. They immunized mice with a single dose of either Fim 2 and 3, FHA, or PT. After 3 weeks mice were bled, and the sera from the individuals within the groups pooled. Similar groups of mice were intranasally infected to compare the ability of the antigens to induce antibodies preventing lung infection with *B. pertussis.* As shown in Table 3, immunization with 10 μg of all three antigens prevented lung infection; the resultant sera also inhibited adhesion of *B. pertussis* to Vero cells. At lower antigen concentrations, however, antisera to FHA and Fim 2 and 3 were apparently more effective in preventing adhesion to Vero cells, whereas immunization with PT was more effective in preventing lung infection. ELISAs indicated that the antisera were specific for the appropriate antigen with negligible cross reaction (data not shown).

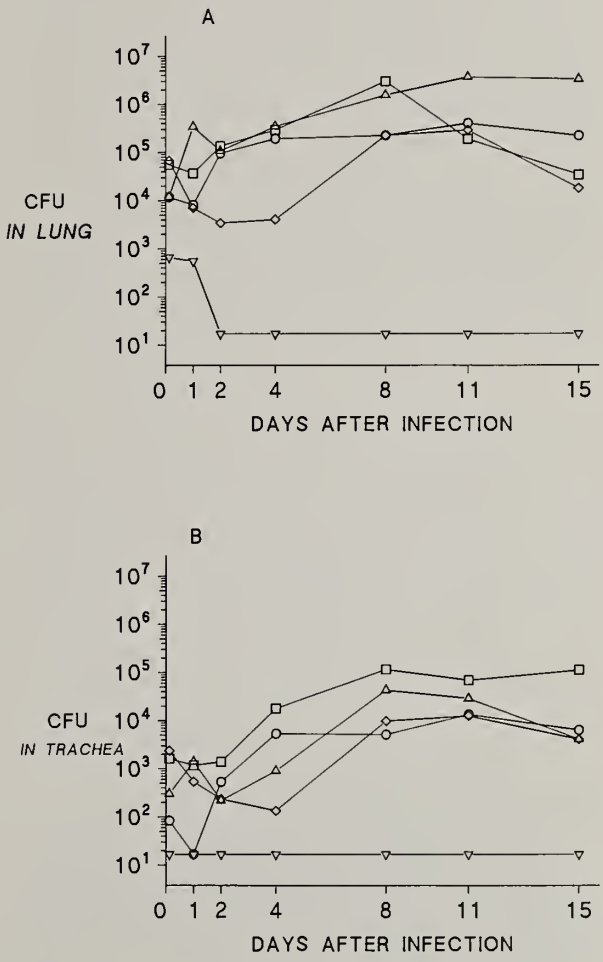

FIG. 5. Colonization of (A) lungs and (B) trachea of mice by various strains of *B. pertussis*. Mice were infected intranasally with 10^6 bacteria; at appropriate times after infection, lungs and tracheas were isolated from groups of mice and the CFU in the organs determined. Wellcome 28, wild type (□); B52, Fim$^-$ (o); 1216 PT$^-$ (◊); B7, FHA$^-$ (Δ); Tn99, Vir$^-$(∇).

Involvement of fimbriae in infection of the respiratory tract of the mouse. The results of Table 3 and previous data on the serospecific protection of mice by purified fimbriae (19) led us to look at the role of fimbriae in colonizing the respiratory tract of the mouse. Mice (6 to 7 weeks old) were intranasally infected with various *B. pertussis* strains (50 μl containing approximately 10^6 bacteria). At appropriate times, groups of three mice were sacrificed, and the extent of colonization of the lungs and tracheas determined. As shown in Fig. 5A, the Vir$^-$ strain was incapable of effectively colonizing the lungs and was not detected 2 days after infection. In contrast, the other strains effectively colonized the lungs. Similarly, the Vir$^-$ mutant was unable to establish an infection in the trachea, but all other strains colonized effectively. Those mutants unable to produce fimbriae did, however, appear to be at an initial disadvantage (Fig. 5B).

Colonization of the lungs of adult mice is not a satisfactory model for whooping cough in the child, and colonization of the trachea in such experiments could be subject to re-infection from the lungs. The researchers next looked at the ability of *B. pertussis* strains to infect the nasal cavity of infant mice (5 to 7 days old). The results, expressed in Fig. 6, are preliminary since only two mice were examined at each point. Again, however, it is clear that although the Vir$^-$ strain was unable to establish infection, all other strains could colonize the nasal cavity.

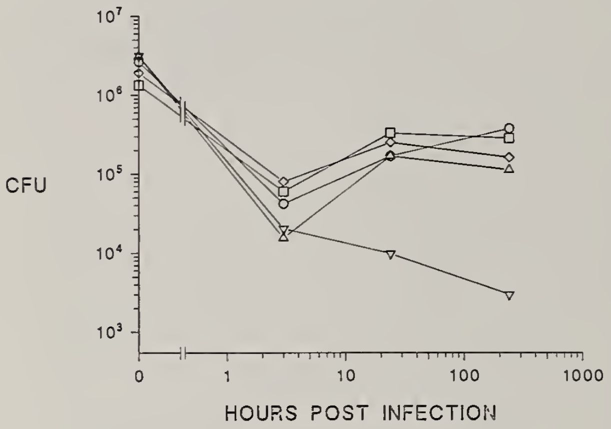

FIG. 6. Colonization of the nasal cavities of infant mice with various strains of *B. pertussis*. Mice were infected intranasally with approximately 10^6 bacteria; at appropriate times after infection, the bacteria in the nasal cavity were enumerated as described in the text. Symbols are as in Fig. 5.

DISCUSSION

B. pertussis fimbriae, Fim 2 and Fim 3, are polymers assembled from monomeric subunits which have molecular weights of 22,500 and 22,000, respectively (18) and show 60% amino acid sequence homology (13, 14). Nonhomologous regions within the sequences presumably account for the serotype specificity which distinguishes Fim 2 and Fim 3. The two fimbrial filaments show close structural similarity (21); this is reflected by their similar conformational stability in Gnd HCl. At neutral pH, both fimbrial types are stable physically and immunologically up to at least 6-M Gnd HCl, even at temperatures of 80°C (data not shown). This robustness is similar to that of *Escherchia coli* Type 1 pili which, at neutral pH and 37°C, requires saturating Gnd HCl (≥ 8.3 M) to dissociate (5).

Aggregation of the individual fimbrial polymers into paracrystalline bundles at pH 7.2 may cause the stability of Fim 2 and 3 at this pH (21). At pH 10.5, however, fimbriae do not form bundles and can undergo a two-stage transition (consisting of a minor component between 0- and 1-M Gnd HCl and a major structural change between 3- and 5-M Gnd HCl) upon exposure to increasing concentrations of Gnd HCl. For the first stage of transition, the decrease in fluorescence intensity indicates increased solvent exposure of the fluorophore(s) which, together with increased binding to antibodies directed against denatured forms of the protein, suggests a minor adjustment of the fimbrial structure. Interestingly, gel filtration of Fim 2 and 3 after treatment with 1-M Gnd HCl showed a slight retardation when compared to native fimbriae (data not shown). This transition may represent a modification of the tertiary structure of the subunits, resulting in a change in fimbrial hydrodynamic shape or size. Alternatively, a structurally important fimbrial-associated protein, distinct from the major subunit, may be dissociated at low Gnd HCl concentrations. Similar proteins have been identified for fimbriae of other species (12, 17). Minor proteins with molecular weights of < 16,000, which may correspond to fimbrial-associated proteins, are occasionally observed in FPLC traces of dissociated Fim 2 and 3. The loss of an associated protein may result in cleavage of the fimbriae at certain infrequent points along the polymer length. Concomitant exposure of a small number of tyrosine residues to solvent and of epitopes reacting with antibodies to denatured fimbriae may ensue.

Between 3- and 5-M Gnd HCl, Fim 2 and 3 appears to display a single step dissociation into monomeric subunits. CD spectra indicate that, in addition to depolymerization, the fimbriae lose their secondary/tertiary structure. This apparent dissociation demonstrates that both Fim 2 and Fim 3 have the same transition point for dissociation, which implies that the polymeric states of Fim 2 and Fim 3 are maintained by similar subunit-subunit interactions which probably involve the homologous amino acid sequences of the subunits. The structural similarity of Fim 2 and Fim 3 was also inferred from the equivalent loss of reactivity to monoclonal antibodies to either fimbria during denaturation of Fim 2 and 3. (Two antibodies to Fim 2 and three to Fim 3 were studied; not all data are shown.) The complete loss of reactivity to monoclonal antibodies in 5-M Gnd HCl indicates that the sero-specific epitopes are conformational; it cannot as yet be determined whether this reactivity depends on the protein's tertiary or quaternary structure. Some of this information should become available from studies currently under way at CAMR to scan overlapping peptides derived from the complete amino acid sequences of Fim 2 and Fim 3 for reactivity with various antibodies.

Several permutations of renaturing conditions have been used in an attempt to reassemble denatured/dissociated fimbriae into structures which are visible in the electron microscope as fimbrial in nature and which also react with antibodies to native fimbriae. Most of the renaturing conditions induced some loss of epitopes specific to the denatured form of the fimbriae; this indicated some refolding but failed to produce any fimbrial-like structure. This finding is consistent with difficulties in attempts to polymerize cloned *B. pertussis* fimbrial subunits (24). In contrast, *E. coli* fimbriae which were dissociated at pH7.2 could be reassembled (5). It is not known whether the irreversible nature of the dissociation of *B. pertussis* fimbriae is induced by high Gnd HCl concentrations, high pH, or a combination of both. Reassembly might possibly have been achieved if separate preparations of Fim 2 and Fim 3 had been employed rather than a mixture of both fimbriae. The failure to renature/reassemble fimbriae also could be due to loss of a fimbriae-associated protein involved in scaffolding or in assisting polymerization of subunits.

No clear role in the pathogenesis of pertussis can as yet be ascribed to fimbriae. The data presented here confirm previous reports that FHA is the major adhesin for *B. pertussis* in in vitro cell culture assays (16, 23, 25). In contrast, fimbriae do not appear to mediate this adhesion and may in fact reduce the extent of adhesion perhaps by steric inhibition of FHA-mediated attachment. Nevertheless, antibodies to fimbriae, along with those to FHA and PT, do inhibit the adhesion of *B. pertussis* to Vero cells. Immunizing mice with low doses of FHA and fimbriae induced antibodies which (i) inhibited adhesion of *B. pertussis* to Vero cells and (ii) protected the mice against respiratory infection with *B. pertussis*. The correlation of protection against lung infection with inhibition of adhesion to in vitro cell cultures suggests that an in vitro cell adhesion inhibition assay may supplement, and indeed supersede, the mouse intranasal protection test for vaccine potency. Mice would simply be immunized with test vaccine, and the antibody levels in the resultant sera determined by ELISA. Functional antibody levels could be determined by the inhibition of adhesion of *B. pertussis* to Vero cells and the neutralization of the toxic effects of PT for Chinese hamster ovary cells.

B. pertussis fimbriae might play a more active role in the infection of laboratory animals and humans when the infecting organisms have to encounter such defense mechanisms as beating cilia and mucus blanket flow. Results suggest that infection of the mouse respiratory tract, particularly the trachea and nasal cavities, is—unlike the infection of in vitro cell cultures—not solely dependent on FHA but is a multifactorial process involving fimbriae, FHA, and possibly PT. Strains deficient in fimbriae and FHA may be at an initial disadvantage in colonizing the trachea and nasal cavities. Other studies using FHA-deficient mutants have shown that FHA is also required for the colonization of the upper respiratory tract of the rabbit (16, 22) and mouse trachea (11), but not the lungs (11, 22). These results, as well as those reported here, are complicated by the fact that the upper respiratory tract can be subject to colonization from a prevalent lung infection.

The role of fimbriae in infection thus remains obscure. It is tempting to speculate that the species tropism exhibited by *B. pertussis* and *B. bronchiseptica* is due to differences in their fimbrial structures (15), since both organisms possess FHA. Perhaps fimbriae are responsible for the bacteria's initial attachment on the respiratory epithelium. Extending current studies with baboon tracheal organ cultures to human tissues should define a function for *B. pertussis* fimbriae.

Results from these studies confirm that even in infection models where fimbriae are not primary adhesins, antibodies directed against fimbriae prevent attachment of *B. pertussis* to target cells: this is the perceived function of antifimbrial antibodies induced by vaccination. The dissociation/denaturation studies clearly show that such antibodies should be directed against native fimbriae, since those induced by certain peptides or denatured fimbriae do not react with the native structures. Further analyses of the functional epitopes of *B.*

pertussis fimbriae subunits are currently under way, as is an analysis of the role of recently identified putative adhesive tip proteins (Williems, this volume).

ACKNOWLEDGMENTS

The authors wish to thank Dr. L. A. E. Ashworth for discussing and critically evaluating this manuscript and Miss R. Greenaway and Mrs. A. Bennett for typing the manuscript.

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DISCUSSION

DR. ULLMANN: It reminds me of very, very old experiments we did 25 years ago some of which were published. When you dissociate a protein in guanidine, depending on the protein, but a big protein never comes back to the native structure after dialysis, but if after guanidine you put the protein in 8 M urea and then renature it using adequate buffers, you can recover practically 100 percent activity; but never from guanidine directly.

DR. ROBINSON: We have tried that and it did not work as well.

DR. SHAHIN: If you have an immune serum or a mouse immunized with fimbriae, do you think that the reason that a decreased bacterial recovery is due to steric hindrance, for instance, with other adhesion mediating molecules or do you think that you are enhancing bacterial clearance by phagocytosis?

DR. ROBINSON: We have not really looked at that. I suspect that steric hindrance may be of major importance but we do not know about all the clearance mechanisms.

DR. STEVEN: What is the evidence that the 40 kDa minor component of your fimbrial prep is actually specifically associated with fimbriae and not some minor contaminant that is still in there?

DR. ROBINSON: I think that Frits Mooi has more data on this than I have but basically the data are that it is a *vir* regulated protein and it is not present if the cells do not produce fimbriae. It is certainly distinct from the 40 kDa major outer membrane protein. If you look at the two proteins on gels, they are totally different. So obviously it could be something that is stuck to the fimbriae but I think Frits Mooi has probably got or will get some genetic evidence that suggests that it is related to fimbriae.

Repeat Sequence Motifs Constitute the Immunodominant Regions of the P.69 Protein, Pertactin from *Bordetella pertussis:* Comparison with Repeat Sequences from *Bordetella parapertussis* and *Bordetella bronchiseptica*

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As previously characterized by Charles et al. (2), the outer membrane protein P.69 from *Bordetella pertussis* can contribute to the protection of mice against an intracerebral (9) or aerosol (11) challenge of *B. pertussis*. Furthermore, monoclonal antibodies (MAbs) against either P.69 or the immunologically related protein P.68 expressed by *Bordetella bronchiseptica* (9) can confer passive protection against a homologous challenge of *B. pertussis* or *B. bronchiseptica*, respectively. These observations suggest that the P.68 and P.69 antigens are important in the protective immune response to the diseases caused by the *Bordetellae*.

Recent studies demonstrated that P.69 binds to mammalian cells and that this binding is mediated, at least in part, by an Arg-Gly-Asp sequence present within the P.69 sequence (2, 5). During the course of infection, P.69 may be involved in the attachment of *B. pertussis* to the host target cells, perhaps by binding to integrins on the surface of macrophages and leucocytes. Thus, the name "pertactin" has been proposed for this protein (5; see Leininger et al. this volume).

This study used a combination of approaches to map the regions of pertactin which react with MAbs. Thus (i) recombinant plasmids expressing various fragments of P.69 and its precursor P.93, (ii) conventionally synthesized peptides, and (iii) peptides synthesized by the "Pepscan" technique (which involves the synthesis of 94 overlapping hexameric peptides on solid supports [3]) were reacted with a panel of MAbs. The study found that two regions of pertactin which encode the repeat sequences $(Gly\text{-}Gly\text{-}Xaa\text{-}Xaa\text{-}Pro)_5$ and $(Pro\text{-}Gln\text{-}Pro)_5$ appear to be immunodominant as defined by reaction with MAbs. These two repeat sequences are also found in the related proteins P.68 and P.70 from *B. bronchiseptica* and *Bordetella parapertussis*, respectively, although the number of repeating units varies between species.

MATERIALS AND METHODS

Monoclonal antibodies. MAbs BB05 and BB07 (Immunoglobulin G1, IgG1) raised against P.68 from *B. bronchiseptica* (8) and MAbs BPE3 (Immunoglobulin M, IgM), BPD8 (Immunoglobulin G, IgG), and BPE8 (IgG1) have been described previously (1). The MAbS D5E9, E4D7, F6E5, and E4A8 (all IgG1) were generated by injecting BALB/c mice with two doses of pertussis whole-cell vaccine followed by one i.p. injection of a semi-purified preparation of filamentous hemagglutinin from *B. pertussis* 3 days before spleen fusion.

Construction of α-peptide fusions to pertactin. The 93-kDa open reading frame encoding the P.93 antigen (2) was expressed in *Escherichia coli* as a series of fusions to the α-peptide of *lac Z* using the pUC family of cloning vectors (7, 13). For more details of the construction, see Charles et al. (submitted for publication to Proc. Natl. Acad. Sci. USA).

Peptide synthesis. Large peptides were synthesized using an adaptation of Merrifield's method (6) as described by Houghten (4). The sequence of peptide 683 is: Ala-Pro-Gln-Pro-Gly-Pro-Gln-Pro-Pro-Gln-Pro-

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* Presenting author.

Pro-Gln-Pro-Gln-Pro-Glu-Ala-Pro-Ala-Pro-Gln-Pro-Pro-Ala-Gly-Arg-Glu-Leu-Ser-Cys. Peptide 684 is: Ala-Gly-Arg-Glu-Leu-Ser-Ala-Ala-Ala-Asn-Ala-Val-Val-Asn-Thr-Gly-Gly-Val-Gly-Leu-Ala-Ser-Thr-Leu-Trp-Tyr-Ala-Cys. Peptide 685 is: Thr-Leu-Trp-Tyr-Ala-Glu-Ser-Asn-Ala-Leu-Ser-Lys-Arg-Leu-Gly-Glu-Leu-Arg-Leu-Asn-Pro-Asp-Ala-Gly-Gly-Ala-Trp-Gly-Arg-Gly-Cys.

Hexameric peptides, overlapping by one residue, were synthesized on solid-phase polyethylene pins as described by Geysen et al. (3). Peptide reactivity to the MAbs was determined by incubating the pins in antibody for 1 to 2 h or overnight, followed by washing and incubation with peroxidase-conjugated goat anti-mouse antibody. Enzyme reactivity was determined by incubating the pins in *ABTS* substrate and measuring the A_{420} of the solution using a Titretek Multiscan MC1100. Pins were sonicated for 30 min at 65°C in 0.1 M Na_2HPO_4, 0.1% SDS, 0.01 M β-mercaptoethanol to remove bound antibody and stain complexes before incubation in subsequent reactions.

RESULTS

Use of *E. coli*-expressed P.69 pertactin proteins to define immunologically reactive domains. A series of plasmids were constructed which expressed the P.93 precursor as a nested set of overlapping deletions (Fig. 1). The proteins expressed by these plasmids were analyzed for their ability to react with a set of MAbs known to recognize pertactin. In Western blotting, the carboxy-terminal fusion proteins PST67 and PVU248 failed to react with either a rabbit polyclonal antibody or any of the MAbs. This is not surprising, as these constructs encode regions found in the P.93 precursor but not in mature pertactin (Fairweather et al., this volume).

Eight of the nine MAbs (BB05, BB07, E4A8, E4D7, D5E9, BPE3, BPE8, and BPD8) recognized one region of pertactin centered around the Pro-Gln-Pro repeat region (B in Fig. 1). The remaining MAb, F6E5, recognized the SAC273 but not SAL146 proteins, indicating that this MAb reacted with a region encompassing both the Gly-Gly-Xaa-Xaa-Pro repeat region and the Arg-Gly-Asp sequence (A in Fig. 1). Recent work (5) suggests that this Arg-Gly-Asp motif in pertactin does have cell-adhesive properties; and consequently, this region of the molecule may be surface exposed.

Reactivity of MAbs with synthetic peptides. Three overlapping peptides were synthesized corresponding to amino acids 537 to 619 of pertactin (region B in Fig. 1). In addition, a hepatitis B core antigen fusion (HBcAg fusion) provided an overlapping sequence containing residues 537 to 566 (M. Romanos, personal communica-

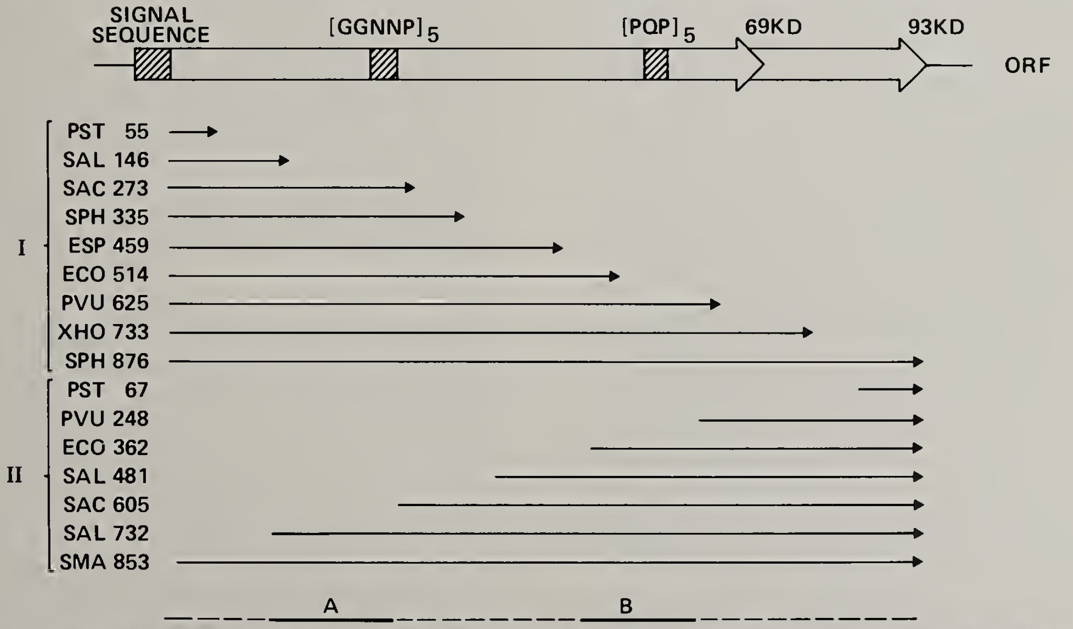

FIG. 1. The overlapping amino- and carboxy-terminal deletions of pertactin generated in the pUC family of cloning vectors are shown. The small arrows represent the extent of expression of each clone. The fusion protein nomenclature indicates the composition of each recombinant fusion; thus, PST55 is a fusion protein which has 55 amino acids of pertactin and terminates at a *PstI* site in the sequence. Series I fusions have a fixed amino terminus; series II fusions have a fixed carboxy terminus. The large open arrow represents the P.93 ORF with the putative signal sequence and repeat motifs shown as hatched boxes. Regions A and B represent the minimal areas in which MAb binding was detected.

```
505
 *
 TLANKDGKVD IGTYRYRLAA NGNGQWSLVG

535
 *
 AKAPPAPKPA PQPGPQPPQP PQPQPEAPAP

565
 *
 QPPAGRELSA AANAAVNTGG VGLASTLWYA

595
 *     ▾
 ESNALS KRL
```

FIG. 2. Sequence of region of pertactin synthesized as a series of overlapping hexamers. Peptide 1 is Thr(505)-Asp(510); peptide 2 is Leu(506)-Gly(511); etc. The arrow after Ser-600 marks the position of cleavage of the P.93 precursor to form P.69.

tion). Reactivity of MAb BB05 occurred within the HBcAg fusion and with peptide 683. MAbs BB07, E4A8, and E4D7 bound only to peptide 683. MAb BPE3 bound only to peptide 685 and not to the overlapping peptide 684, thus suggesting that the epitope is located within the last 23 residues of peptide 685 or at the junction of peptides 684 and 685.

Precise mapping of epitopes using Pepscan. To map the epitopes for the monoclonals precisely, a series of overlapping peptides were synthesized as described by Geysen et al. (3) and their reactivity to a series of MAbs determined using ELISA. Ninety-four hexameric peptides were synthesized, covering residues 505 through 603 of P.69 pertactin (Fig. 2). Thus, peptide 1 is Thr(505)-Asp(510) and peptide 94 is Ala(598)-Leu(603). Three of the monoclonals (BB07, E4A8, and E4D7) bound to only one region around peptide 60 (residues Ala-Pro-Gln-Pro-Pro-Ala-Gly-Arg). Although the peptides recognized by these MAbs were not all identical, they did overlap, indicating that these MAbs have shared, but distinct, epitopes. MAb BB05 recognized only peptide 43 (Pro-Gly-Pro-Gln-Pro-Pro)(Fig. 3), which locates the epitope in the region of the (Pro-Gln-Pro)$_5$ repeats (Fig. 1). MAb BPE8 recognized several peptides. These peptides were clustered in distinct groups, however, centered around peptides 20 to 34, 41 to 43, 53 to 55, 63 to 65, and 74 to 76 (Fig. 3).

The Pepscan peptides failed to give any reaction with the IgM BPE3 and the IgG BPD8. However, MAb BPE3 reacted with the larger synthetic peptide 685, thus localizing its binding to the C terminus of pertactin.

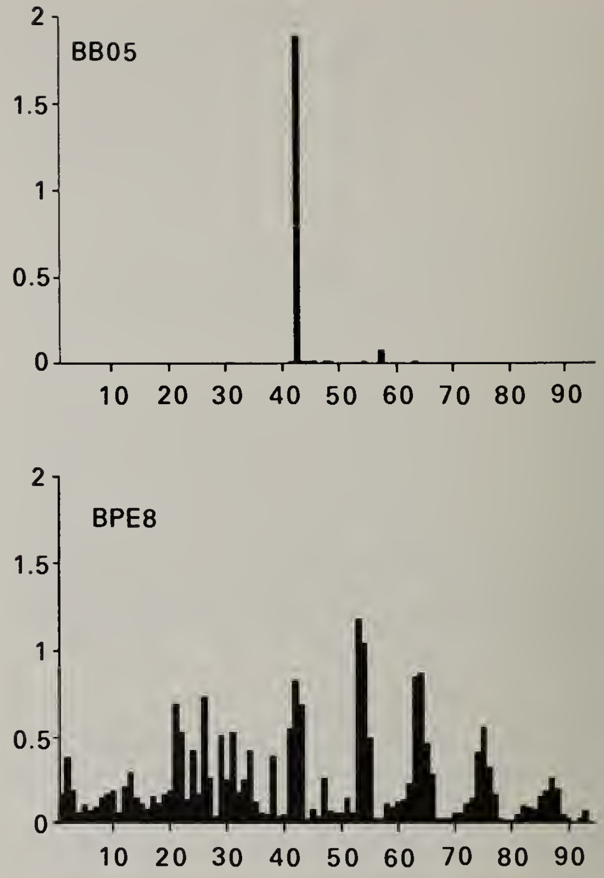

FIG. 3. Reactivity of MAbs BB05 and BPE8 to the series of hexameric peptides synthesized with Pepscan technology. The numbering system is described in the text and in Fig.2. Pins were reacted with MAbs as described in the text; the A_{420} of each pin is given on the y-axis. The complex pattern of reactivity shown by BPE8 is probably due to the MAb recognizing a conformational epitope.

Comparison of the pertactin repeat motifs with those of P.68 and P.70. A recent study cloned and determined the DNA sequence of the genes encoding the P.68 and P.70 antigens from *B. bronchiseptica* and *B. parapertussis* (L. J. Li et al., submitted to Molecular Microbiology). A computer program, ALIGN, individually aligned the deduced protein sequences with that of pertactin (12). All three sequences showed a high degree of similarity, with the only significant differences occurring in the number of reiterated units within the repeat sequence blocks (Fig. 4). All three sequences demonstrate the presence of the binding sites for the MAbs BB05, BB07, E4D7, E4A8, D5E9, and BPE3. We are currently

232 545

Bp: GGAVP GGAVP GGAVP GGFGP GGFGP // --- - $\overline{\text{PQP}}$ G $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ --- --- --- --- Q PEAPA $\overline{\text{PQP}}$ PAGRELSA

Bpp: GGAVP GGAVP GGAVP GGFGP ----- // $\overline{\text{PQP}}$ G $\overline{\text{PQP}}$ G $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ PQR Q PEAPA $\overline{\text{PQP}}$ PAGRELSA

Bb: GGAVP GGAVP ----- GGFVP ----- // $\overline{\text{PQP}}$ G $\overline{\text{PQP}}$ G $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ $\overline{\text{PQP}}$ --- --- PQR Q PEAPA $\overline{\text{PQP}}$ PAGRELSA

FIG. 4. Alignment of the repeat sequence motifs of the P.68, P.69, and P.70 pertactin species. The sequence Bp is from *B. pertussis*, Bpp from *B. parapertussis*, and Bb from *B. bronchiseptica*. In *B. pertussis* P.69, there are five repeats of both the Gly-Gly-Xaa-Xaa-Pro and Pro-Gln-Pro motifs. In *B. parapertussis* there are four repeats of Gly-Xaa-Xaa-Pro and nine repeats of Pro-Gln-Pro. In *B. bronchiseptica*, there are three repeats of Gly-Gly-Xaa-Xaa-Pro and seven of Pro-Gln-Pro.

determining the reactivity of these MAbs to synthetic peptides corresponding to the P.68 and P.70 sequences.

DISCUSSION

Brennan et al. have identified the outer membrane protein pertactin (P.69) as an agglutinogen; other researchers have demonstrated it to be a protective antigen (9, 11). Using a combined genetic and synthetic peptide approach, the present study mapped those regions of the molecule which are immunologically important. These immunodominant parts of the protein coincide with repeat motifs present in the protein sequence. By elucidating the DNA sequence of the genes encoding the related P.68 and P.70 proteins from *B. bronchiseptica* and *B. parapertussis*, respectively, the studies also demonstrate that these repeat elements are the major regions of variability among the three proteins. This variability exists in the number of repeated units rather than in the composition of repeats.

For pertactin, MAbs were mapped to the following sequences: BB07, E4D7, and E4E8 bound to the sequence around Ala-Pro-Gln-Pro-Pro-Ala-Gly-Arg; and the protective MAb BB05 bound to the sequence Pro-Gly-Pro-Gln-Pro-Pro. MAb BPE3 was found to bind at the last 11 amino acid residues at the extreme carboxy terminus of the molecule (see Fairweather et al. this volume for the C terminal mapping data). The reactivity of the protective MAb BPE8 was fairly complex (Fig. 3), which, combined with its poor binding with denatured pertactin in Western blotting, suggests that it may recognize conformational epitopes. The linear residues identified by the Pepscan analysis of BPE8 could contribute to the conformational binding of this MAb to pertactin. A similar technique identified discontinuous linear peptide sequences which form a conformational epitope. Parry et al. characterized the sequences recognized by one neutralizing MAb for the foot-and-mouth disease virus (10) and found the epitope to be composed of two linear, but discontinuous, sequences.

The finding that one of the MAbs, F6E5, binds around the Arg-Gly-Asp-(Gly-Gly-Xaa-Xaa-Pro) region could be significant since the Arg-Gly-Asp-mediated attachment of pertactin to eukaryotic cells may be of biological importance in the disease state mediated by *B. pertussis*, and antibodies that block binding may also block attachment and might be expected to be protective. This hypothesis is being tested in animal models.

Aligning the deduced sequences of the *B. bronchiseptica* P.68 and *B. parapertussis* P.70 proteins with pertactin demonstrates a high degree of homology. All three proteins possess the linear sequence Pro-Gly-Pro-Gln-Pro-Pro which constitutes the binding sites for the protective MAb BB05. Pure P.68 and P.70 proteins are being prepared for use in ELISAs against the panel of MAbs to determine how much cross-reactivity occurs, particularly for the protective conformational MAb BPE8.

ACKNOWLEDGMENTS

We thank Tina Silva for typing the manuscript, Hugh Spence for oligonucleotides, and Dick Campbell and Mike Francis for peptides.

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DISCUSSION

DR. LOCHT: What about the fusion?

DR. CHARLES: No, we have not done that.

Characterization of the Major Outer Membrane Porin Protein of *Bordetella pertussis*

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The outer membrane of gram-negative bacteria contains a specialized class of proteins, porins, which function as water-filled channels for the diffusion of hydrophilic molecules. Most of the porins which have been characterized have monomer molecular masses between $M_r = 30{,}000$ and $M_r = 50{,}000$, with the functional units organized in the outer membrane as trimers of porin subunits (3). Porin proteins form simple diffusion pores which separate molecules on the basis of size and often with charge specificity (12). A number of substrate-specific porins have also been identified, including Protein P of *Pseudomonas aeruginosa* (13) and LamB of *Escherichia coli* (4), which contain binding sites for phosphate and maltose-maltodextrin, respectively. Since porins of *Neisseria meningitidis* are prominent outer membrane components which exhibit antigenic diversity among different strains, they have been used as serotyping antigens (10). *Salmonella typhimurium* porins have been shown to play a role in bacterial pathogenicity since they decrease macrophage viability and deplete complement activity (11) and are thought to induce the synthesis of protective antibodies against disease (18).

It has recently been shown that the porin of the human pathogen *Bordetella pertussis* is present in a naturally crystalline state in certain strains, a property that has so far been shown to be relatively unique to this gram-negative bacterium (15). Also, studies using black lipid bilayers have indicated that a $M_r = 40{,}000$ heat-modifiable protein from *B. pertussis* is a porin which forms an anion-specific channel (1). This report presents a method for the large-scale purification of porin, describes the cloning and sequencing of the porin gene, and shows that antibodies can be elicited against both the native and monomeric forms of this protein.

MATERIALS AND METHODS

Reagents and chemicals. Zwittergent 3-14 detergent was purchased from Calbiochem-Behring (La Jolla, Calif.) and Triton X-100 detergent from Research Products International Corporation (Elk Grove Village, Ill.). Restriction enzymes, T4 DNA ligase, and Random Primers DNA Labeling System were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The DNA 5′ Endlabeling Kit was ordered from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). We obtained [α^{32}P]-dCTP (800 Ci/mM) and [γ^{32}P]-ATP (6000 Ci/mM) from New England Nuclear Corp. (Boston, Mass.). Oligonucleotides were synthesized at Pharmacia Fine Chemicals (Piscataway, N. J.) or Genosys Biotechnologies, Inc. (Woodlands, Tx.); some were provided by Elizabeth Leininger, Laboratory of Cellular Physiology, Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, Md.). Sequenase® DNA Sequencing Kit was purchased from United States Biochemical Corp. (Cleveland, Ohio). Protoclone λgt11 system and phosphorylated *Eco*RI linkers were obtained from Promega Biotech (Madison, Wis.).

Bacterial strains and plasmids. *B. pertussis* strains 347 and 10901 were obtained from the Laboratory of Pertussis Collection, Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, Md.). Cells were cultured as previously described (9). *B. pertussis* 10901 cells were grown in the National Institutes of Health pilot plant fermentor under the super-

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* Presenting author.

TABLE 1. Purification of porin from *B. pertussis* 10901

Purification step	Total protein (g)[a]	Porin (g)[b]	Recovery (%)
1. Starting material (700 g wet weight)	47.0	9.2	100
2. Cell envelopes	6.0	2.4	26
3. 2% Triton X-100 soluble extract	1.5	N D	--
4. 2% Triton X-100 insoluble pellet	4.0	1.8	20
5. 5% Zwittergent[c] soluble extract	2.5	2.0	22
6. DEAE-Sephacel (peak 1)	0.7	0.7	8

[a] Protein concentration was estimated by spectrophotometric analysis at 276 mm.

[b] The amount of total porin protein was estimated by densitometric analysis of fraction separated by sodium dodecly sulfate-polyacrylamide gel electrophoresis.

[c] Protein recovery represents that total from six consecutive extractions with this detergent.

ND, Not detectable.

vision of Jossi Shiloach. *E. coli* strain Y1090 was obtained from Promega. Library efficiency DH5α competent cells were purchased from Bethesda Research Laboratories, and the pUC18 *Bam*HI/BAP vector was purchased from Pharmacia.

Porin purification. Porin from strain 10901 was isolated as shown in Table 1. Approximately 100 liters of *B. pertussis* 10901 cells (700 wet weight) were grown in submerged culture, centrifuged and resuspended, in 4 liters of 10 mM of Tris-HCl pH 8.0 containing 5 mM of $MgCl_2$ and 12 μg of DNase per ml. The bacteria were passed through a French Press (Manton Gaulin homogenizer 15M-8TA). Cellular material was then centrifuged at 23,000 x *g* to pellet cell debris and unbroken cells. This supernatant (3 liters) was then centrifuged at 100,000 x *g* for 1 h to pellet cell envelopes. The cell envelopes were suspended in 200 ml (1/15 vol) of 2% Triton X-100 detergent in 10 mM of Tris-HCl pH 8.0 for 1 h at 37°C and then centrifuged at 100,000 x *g* for 1 h. The pellet was resuspended in 200 ml (1/15 vol) of 20 mM of Tris-HCl pH 8.0 containing 5% Zwittergent 3-14 and 0.5 M of $CaCl_2$, incubated for 1 h at 37°C, and again centrifuged at 100,000 x *g* for 1 h. Samples of the Zwittergent 3-14 extract were precipitated with four volumes of ice cold ethanol at -20°C overnight. Precipitated proteins were collected by centrifugation at 23,000 x *g* for 30 min and solubilized in 1/4 the original volume using 50 mM Tris-HCl pH 8.0 containing 0.5% Zwittergent 3-14 and 10 mM of EDTA. This material was applied to a DEAE-Sephacel (Pharmacia) anion exchange column (2.5 x 6 cm) and washed with the above buffer. The column was then eluted with a 0 to 1 M NaCl gradient in column buffer. Two peaks were obtained at an initial salt concentration of 0.15 M NaCl (peak I) and 0.4 M NaCl (peak II). Most of the porin protein was present in peak I.

Construction and screening of gene banks. Genomic DNA from *B. pertussis* 347 was sheared with passages through a 30-gauge needle such that most of the DNA fragments were between 2 and 8 kilobases (Kb). The sheared DNA was methylated with *Eco*RI methylase to remove endogenous *Eco*RI sites. The DNA fragments were made flush with T4 polymerase and then ligated to *Eco*RI linkers using T4 DNA ligase. Excess linkers were removed by *Eco*RI digestion and subsequent passage through low melting point agarose. The treated DNA was then ligated into λgt11 arms and packaged into phage heads using the Packagene system (Promega). The library was amplified to a final concentration of 1 x 10^{11} PFU/ml and was then used to infect *E. coli* strain Y1090 and subsequently plated onto Luria-Bertani medium plates. After growth at 42°C, the plates were overlayed with IPTG-saturated nitrocellulose filters (Schleicher and Schuell) for 20 min. The filters were then removed and dipped sequentially in (i) 0.2 M NaOH and 1.5 M NaCl, (ii) 2 x SSC in 0.4 M Tris pH 7.4, and (iii) 2 x SSC. Filters were baked in a vacuum oven for 1 h at 80°C. Oligonucleotides (oligo-A) were end-labeled using [γ^{32}P]-ATP, and hybridization was performed at 60°C overnight. The filters were then washed three times in 1 x SSPE containing 0.1% sodium dodecyl sulfate (SDS). Positive plaques were selected after autoradiography and purified by repeated screening.

Additional screening of a pUC18 partial gene bank was performed as follows. Chromosomal DNA of BP 347 was digested with the restriction enzyme *Bgl*II which was chosen based on the restriction site map of BpPor1b, a clone selected using the λgt11 library. DNA fragments of approximately 6 Kb were excised from low melting point agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) and inserted into a pUC18 *Bam*HI/BAP vector (Pharmacia) with compatible cohesive ends. The recombinants were transformed into *E. coli* DH5α competent cells and then plated onto Luria-Bertani medium plates supplemented with X-GAL and IPTG. White colonies were selected and inoculated onto duplicate agar plates containing nitrocellulose filters. The plates were incubated at 37°C overnight. The filters were placed on filter paper saturated with 0.5 M NaOH to denature DNA and then neutralized by laying the filters

TABLE 2. Comparison of the predicted amino acid sequence of *B. pertussis* porin genewith published porin sequences

Bacterial porins		Amino acid overlap (position)	Homology (%)
N. gonorrhoeae	PIA	203 (1-201)	29.6
N. meningitidis	Class 1	277 (1-271)	24.2
E. coli	OmpC	259 (29-284)	17.4
E. coli	PhoE	201 (64-264)	18.9

The range of amino acids showing homology is designated, and their corresponding position in the protein is given in parentheses. Homology was determined using the National Institute of Health Protein Identification Resource (PIR).

on paper saturated with 1.5 M NaCl and 0.5 M Tris, pH 8.0. The filters were air dried, baked at 80°C for 1 h, prewashed in 50 mM Tris, pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1% SDS, and then hybridized with the [$\gamma^{32}P$]-ATP labeled oligonucleotide (oligo B) at 50°C overnight (19).

DNA sequencing. For sequencing the BpPor1b clone, the 2.3-Kb *Eco*RI fragment was excised from λgt11 recombinant phage DNA which was isolated using the Lambda Sorb system (Promega). This fragment was then cloned into the vector pUC18. The 6-Kb BpPor2 fragment was excised with *Eco*RI and *Hin*dIII and cloned into pUC18. DNA from both clones was isolated by the alkaline lysis method (19). Restriction endonucleases selected after restriction mapping were used to generate smaller DNA fragments which were then subcloned into pUC18 for further DNA sequencing. Oligonucleotides derived from sequencing results, as well as pUC18 forward and reverse primers (Promega), were used as primers for double-stranded sequencing with Sequenase® (USB). The sequence of some DNA fragments were also confirmed by Lark Sequencing Technology, Ltd. (Houston, Texas). Computer analysis of the sequence was performed using the National Institutes of Health Protein Identification Resource.

Analytical procedures. Porin samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (16) using 4 to 20% polyacrylamide gradient gels containing SDS (ISS-Enprotech, Hyde Park, Mass.). Samples containing 0.5 M $CaCl_2$ were dialyzed before SDS-PAGE. To obtain the porin monomer, the sample was boiled for 10 min prior to electrophoresis or, to visualize the oligomeric form, the sample was held at room temperature. Gels were stained with Coomassie blue to visualize the proteins. Western blotting (immunoblotting) was performed as previously described (5) using sera from mice immunized twice with 20 µg of purified porin. Proteins were assayed using the BCA protein assay (Pierce Chemical Co., Rockford, Ill.) and endotoxin was determined using the Limulus Amoebocyte Lysate assay (8).

For amino acid analysis, a sample of DEAE-purified porin (peak I) was dialyzed extensively against 1 M NH_4OH and chromatographed on Sephadex G-50 fine grade (Pharmacia) using 1 M NH_4OH to remove detergent. Material eluting just after the void volume was used to determine the amino acid composition and NH_2-terminal amino acid sequencing. NH_2-terminal amino acid sequencing was performed using a microsequencer (Applied Biosystems, Inc.) with an on-line PTH amino acids analysis system (14).

RESULTS

Purification of Porin. An isolation procedure using *B. pertussis* strain 10901 is summarized in Table I. *B. pertussis* 10901 cells were grown in submerged culture, centrifuged, and the bacteria were passed through a French Press (Manton Gaulin Homogenizer 15M-8TA). Cell envelopes were collected by centrifugation and resuspended in 2% Triton X-100 detergent in 10 mM Tris-HCl pH 8.0, which solubilized a number of outer membrane proteins not related to the porin protein. After centrifugation, the pellet was resuspended in 20 mM of Tris-HCl, pH 8.0 containing 5% Zwittergent 3-14 and 0.5 M $CaCl_2$; this extract primarily contained the porin protein. The pellet was extracted a total of six times with Tris buffer containing Zwittergent 3-14 for efficient recovery of porin.

Further purification of porin from Zwittergent 3-14 extracts was accomplished by ion-exchange chromatography using DEAE-Sephacel. Partially purified porin protein was applied to a DEAE-Sephacel anion exchange column, and washed with 50 mM Tris-HCl pH 8.0 containing 0.5% Zwittergent 3-14 and 10 mM EDTA until the 276 nm absorbance of the effluent returned to baseline. The column was then eluted with a 0 - 1 M NaCl

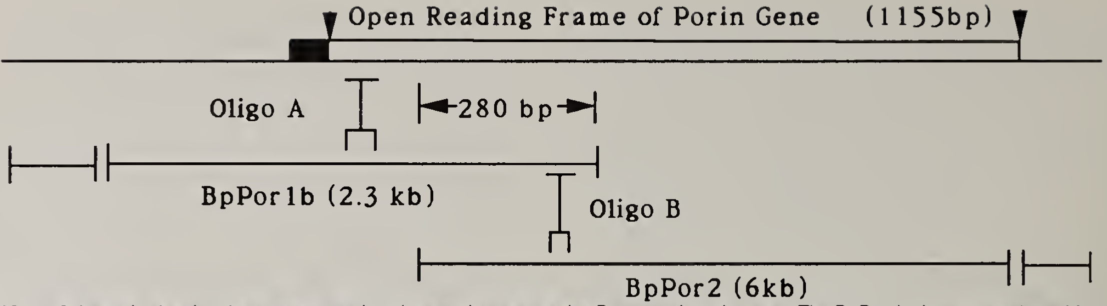

FIG. 1. Schematic showing the strategy used to clone and sequence the *B. pertussis* porin gene. The BpPor1b clone was selected from the λgt11 library using oligonucleotide A (Oligo A) and the BpPor2 clone was selected from a pUC18 library using oligonucleotide B (Oligo B). A 280-bp region of overlap between BpPor1b and BpPor2 is indicated, and the open reading frame designated by these clones is shown as a bar with the filled region representing the region of the signal peptide. The signal peptide cleavage site and the putative termination codon are indicated by arrows.

gradient in column buffer, 3 ml fractions were collected, and absorbance at 276 nm was measured. Two peaks were obtained at an initial salt concentration of 0.15 M NaCl (peak I) and 0.4 M NaCl (peak II). Most of the porin protein was present in peak I and gave a yield of approximately 1.0 mg porin per gram wet weight of cells. A final recovery of 8% of the porin present in the starting material was obtained, as estimated by densitometric analysis of fractions separated by SDS-PAGE (Table I).

Cloning and sequencing of porin. Amino acid analysis of purified porin gave a single NH_2-terminal amino acid sequence which suggested the presence of only one major protein in this preparation. The initial yield of this sequence was calculated to be 75 ± 5%. The NH_2-terminal sequence of the porin was used to design an oligonucleotide (Oligo A = 5′-d[GAC AC(C/G) GGC ATC GGC TA(C/T) AA(C/T)GAC CT(C/G) GAC TTC AAG]-3′) for screening the *B. pertussis* λgt11 library. One clone (BpPor1) was identified, and a 2.3 Kb DNA fragment was specifically detected by Oligo A (Fig. 1). This fragment was subcloned into pUC18 (BpPor1b), partially sequenced, and found to contain a predicted amino acid sequence nearly identical to the NH_2-terminal amino acid sequence determined by biochemical analysis.

A second oligonucleotide (Oligo B = 5′-d[G AGC GCG ATG AAC ACC GTT CGC TAC GA]-3′) was constructed from a region near the 3′ end of BpPor1b and used to screen a pUC18 library containing DNA from strain 347 that had been digested with the endonuclease *Bgl*II. A second clone was identified (BpPor2) which contained a 6-Kb *Eco*RI/*Hin*dIII fragment which hybridized with Oligo B (Fig. 1). Sequencing of BpPor2 demonstrated a region of 280 bp which is identical to the sequence found near the 3′ end of BpPor1b. The BpPor2 fragment also contains a putative termination codon (TAA) that is 1,155 bp downstream from the start codon found in BpPor1b. The entire sequence would encode for a protein of 39,103 daltons consisting of 365 amino acids and a 20 amino acid putative signal peptide. Using a data base search, the *B. pertussis* porin gene was found to have prominent regions of homology with other porin genes (Table 2). The PIA porin from *Neisseria gonorrhoeae* (6), the class I outer membrane protein from *N. meningitidis* (2), and the OmpC (20) and PhoE (21) porins from *E. coli* demonstrate the greatest amount of homology.

Immunogenic properties of *B. pertussis* porin. Mice immunized with porin purified from the avirulent strain 10901 produced antibodies directed against both the 40-kDa monomeric protein (Fig. 2, lane 1) and the multimeric native forms of the *B. pertussis* porin (Fig. 2, lane 2). The lower molecular weight band present in lane 1 appears to result from proteolytic degradation of the monomer. Although the protein is immunogenic, mice immunized with either monomeric or native porin from this avirulent strain were not protected from leukocytosis and death in the aerosol challenge model for pertussis. This result may be explained, in part, by the finding that the antisera produced reacts very weakly with the surface of the 18323 challenge strain. Porin protein purified from virulent strains of *B. pertussis* has not been tested in the aerosol system.

DISCUSSION

The results presented here indicate that *B. pertussis* cells contain one major porin protein found in both virulent and avirulent strains. Its expression is not regulated by the *vir* locus of *B. pertussis* (23, 24) as has been noted for several other outer membrane proteins (5, 7, 25). In fact, about three times more protein can be extracted from the avirulent strain 10901 than from the

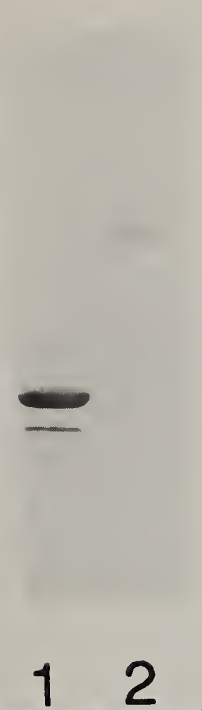

FIG. 2. Immune response to porin. A nitrocellulose filter containing purifed porin monomer (lane 1) or purifed oligomeric porin (lane 2) was incubated with a 1:500 dilution of mouse sera and immunoblotting was performed as described in text.

virulent strain Tohama I. The presence of porin is also not coordinately regulated with the expression of complex lipooligosaccharide since strain 10901, a "rough" mutant which lacks repeating oligosaccharide units on lipooligosaccharide (LOS A) (22), contains about 1.5 times more porin than the avirulent strain Tohama III which expresses LOS A (17). Although the possibility cannot be ruled out that minor porin proteins may be produced by *B. pertussis* or that small differences in the composition of the porins produced by virulent and avirulent strains may exist, the results indicate that only one major porin protein is expressed by *B. pertussis.*

B. pertussis strain 10901 was used for porin purification since it produces the protein in relatively large amounts and is an avirulent mutant strain which does not express virulence-associated outer membrane proteins or toxins that could potentially contaminate the final preparation. Using a purification procedure similar to that described by Armstrong et al. (1), approximately 1 mg of porin was purified per gram wet weight of *B. pertussis* 10901 cells. Extraction of the cell envelopes with 2% Triton X-100 is an important step in this procedure because it essentially solubilizes most of the proteins in the cell envelope except porin, which can then be efficiently extracted with the detergent Zwittergent 3-14. With the possible exception of the first Zwittergent 3-14 extraction which contained some contaminating proteins, successive extractions of the cell envelopes with Zwittergent 3-14 improved the total recovery of porin protein but did not affect the purity of the extract. The need to extract the cell pellet repeatedly may reflect the difficulty in solubilizing the cell envelopes and/or the nature of the interactions between porin and other membrane components such as peptidoglycan and lipooligosaccharide.

The *B. pertussis* porin protein binds to DEAE and is eluted at salt concentrations greater than 0.15 M NaCl. This procedure results in the further purification of porin as detected by the loss of contaminating protein bands in SDS-PAGE as well as a significant separation from endotoxin. In these studies, DEAE-purified porin contained in the first peak eluted by the NaCl gradient contained 0.025 ng of endotoxin per µg of protein, a level that is much lower than that currently allowed for vaccine use in humans.

The gene for the porin was identified from a library of *B. pertussis* DNA inserted into a λgt11 system, using an oligonucleotide probe designed from the NH_2-terminal amino acid sequence of the purified porin protein. A truncated gene corresponding to the NH_2-terminal region of the protein was initially found. Subsequently, a second clone was identified using a pUC18 gene bank which contained the remaining carboxy-terminal portion of the gene. The entire gene encodes for 365 amino acids and a protein with a predicted mass of 39,103 daltons. The *B. pertussis* porin gene has a high degree of homology with other porin genes including the PIA porin of *N. gonorrhoeae* and the OmpC and PhoE porins of *E. coli.* It is also very homologous to the class I outer membrane protein of *N. meningitidis.*

Initial results demonstrated that both purified porin protein and whole bacteria elicit an immune response directed against porin in mice, although the porin protein purified from the avirulent *B. pertussis* strain 10901 did not protect mice against respiratory challenge with virulent *B. pertussis* organisms. Recently, porin has been purified from a virulent *B. pertusssis* strain and the ability of this preparation to function as a protective antigen is being investigated.

B. pertussis porin is capable of being isolated and purified in large amounts and it may be possible to construct and express recombinant forms of this protein. These characteristics make it an ideal candidate for several areas of study, including crystallization and structural studies by electron microscopy and x-ray diffraction, analysis of substrates channeled through this porin as well as potential binding sites for these substrates, and the study of the porin's immunogenic properties.

ACKNOWLEDGMENTS

We thank Scott Stibitz, Sheldon Morris, and David Rouse for assistance in molecular techniques; Robert Boykins for amino acid analysis; and Jossi Shiloach for cultivation of bacteria.

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DISCUSSION

DR. QUENTIN-MILLET: You have alluded to the homology between the points by showing the internal sequence of points from two strains. Have you looked further? Are these points, do they show any antigenic variation or are they constant, the foreign proteins?

DR. BRENNAN: You mean immunologically?

DR. QUENTIN-MILLET: Yes.

DR. BRENNAN: Well, the monoclonal blot I showed suggests that there are common epitopes but suggests there are also different epitopes.

DR. QUENTIN-MILLET: Within *Bordetella pertussis?*

DR. BRENNAN: I do not know.

DR. COWELL: Have you done active immunization of animals for protection or has only passive immunization with the antibodies been done?

DR. BRENNAN: That was active immunization I was alluding to.

DR. COWELL: Did you determine if the antibodies not only react to the foreign protein on a Western, but also in an ELISA to the purified protein? Also, do the antibodies react to the surface of the whole organism?

DR. BRENNAN: Those are good questions. The antibodies will see porin, native porin done on an ELISA plate but the mouse sera did not bind very well to the 18323 strain that is used as the challenge culture.

Outer Membrane Vesicles of *Bordetella pertussis* Are Protective Antigens in the Mouse Intracerebral Challenge Model

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Since it was demonstrated that the *Bordetella pertussis* whole-cell vaccine's (WCV's) potency in the intracerebral (i.c.) mouse protection assay correlated with field protection (8), this test has been mandatory for controlling pertussis WCV. It was subsequently shown that several *Bordetella* antigens are protective when tested in the i.c. mouse protection assay (10, 11, 15, 16). These antigens include toxoided pertussis toxin (PT), filamentous hemagglutinin (FHA), fimbriae, virulent-phase outer membrane proteins (OMPs), and avirulent-phase OMP. With the exception of toxoided PT (PT_x) all other antigens seem to require nonprotective amounts of active PT to be protective in the i.c. test (10, 15). FHA free from PT appears able to protect when given in a two-dose schedule (16).

The action of PT (when given 7 to 14 days before challenge) can be explained in several ways, including synergism, adjuvant activity, or an increase in vascular permeability of the blood-brain barrier. Although the last explanation is most favored, the blood-brain barrier in protected mice becomes permeable around day 4 after challenge, coinciding with the decline in bacterial counts (2, 4). Whatever explanation is correct, the finding that active PT does potentiate the protective activity of purified antigens has prohibited characterization of the major i.c.-protective *B. pertussis* antigens present in WCVs.

This study attempted to re-evaluate these findings by using a PT^- mutant strain and testing the i.c.-protective capacity of a number of antigens. We used BP-TOX6 (14) which had a deletion of the PT operon and tested purified FHA; fimbriae; outer membrane vesicles (OMVs); and 92K, 69K, 38K, and 32K OMPs. Chemically and genetically toxoided PT purified from a tox^+ strain was also tested. The toxoids, FHA, and OMVs all appeared to be i.c.-protective antigens. Serological analysis of mouse sera revealed strong homologous antibody formation in all cases. A reference WCV induced a significant antibody response against the OMPs present in OMV and WCV preparations only.

MATERIALS AND METHODS

Strains and growth conditions. *B. pertussis* strains 134 (atypical lipopolysaccharide (LPS, Fim 3) and 509 (typical LPS, Fim 2) were used to prepare the Netherlands WCV (20). Charles Manclark kindly provided strain Tohama. Strain BP-TOX6 was received from Rino Rappuoli and was described previously (14). Strains W28 and W28 ($69K^-$) were obtained from Gordon Doughan. Strain 18323 was used as the i.c. challenge strain. Mutants from strain 509 were obtained by colony blotting using monoclonal antibodies. After growth for 3 days on Bordet-Gengou agar plates (Difco Laboratories), the colonies were blotted onto nitrocellulose filters and screened for the presence of fimbriae, FHA, and 92-kDa OMP. $Fimbriae^-$ and FHA^- mutants were easily picked up this way. Small-scale liquid growth of *B. pertussis* was carried out in shaking flasks containing Verwey medium consisting of 14 g/L Casamino Acids, KCl 0.2 g/L, starch 1 g/L, KH_2PO_4 0.5 g/L, $MgCl_2.H_2O$ 0.1 g/L, nicotinic acid 0.02 g/L, glutathion 0.01 g/L. For growth into the avirulent C-mode 0.5 g/L nicotinic acid was added. Large-scale fermentor liquid growth was carried out in 40-L, 140-L, or 300-L Bilthoven units in B2-medium under pH and pO_2 control (20). The cells were inactivated by heating at 56°C for 10 min, centrifuged at 3,000 x *g*, and resuspended in saline. Opacity Units (OUs) were detected using a reference standard. Protein determination was carried out with the bicinchoninic acid protein assay reagent (Pierce Chemical Co.) using bovine serum albumin as a standard.

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I.C. mouse potency test. The i.c. test was carried out according to Kendrick et al. (6). Male and female NIH-outbred mice were used (10 to 14 g). Each vaccine was tested in four fivefold dilutions (20 mice/dilution) by intraperitoneal immunization (0.5 ml, containing 1 mg $AlPO_4$). WCVs were tested using 1, 0.2, 0.04, 0.008 OU. Since 1 OU contains approximately 15 µg protein, the final calculations were made for purified antigens on a protein-comparative basis. OMV, PTd, and FHA were tested in 5, 1, 0.2, and 0.04 µg doses. Purified antigens were tested in 25, 5, 1, and 0.2 µg protein doses on the basis of pilot experiments. A reference WCV with standard potency was part of each experiment. The mice were challenged intercerebrally 14 to 16 days after immunization with 10 µl of a suspension of *B. pertussis* 18323 containing 15×10^3 bacteria. This suspension was prepared from a lyophilized culture having an LDSO of $\wedge < 10^3$ bacteria. This culture was grown on Bordet-Gengou plates for 4 days, subcultured on Bordet-Gengou for 24 h, and suspended in 1% Casamino Acids in saline to a concentration of 15×10^5 bacteria/ml. Groups of 12 mice were injected with 15×10^3, 1,500, 300, or 60 bacteria. For calculation, only mice dying from days 4 to 14 were taken into account. Based on the percentage of surviving mice, the potency of the vaccine was calculated by probit analysis. The test has a 95% confidence limit of 55% to 192% of the reference preparation. Vaccine potency was calculated (after probit transformation) by the formula

$$\frac{\text{potency standard}}{\text{potency test}} = \frac{\text{ED 50 test}}{\text{ED 50 standard}}$$

Preparation of OMVs. OMVs were prepared by resuspending bacteria after growth and centrifugation in 10 mM Tris pH 8.0, 2 mM EDTA. Approximately 10 ml of Tris was used to resuspend bacteria from 1 liter of 100 OU/ml of culture. The suspension was sonicated on ice for 10 min. (50% power, 100 W, 50% duty cycle, Branson 250 Sonifier). After centrifugation for 10 min at 10,000 x *g*, the supernatant was pelleted for 1 h at 50,000 x *g*. This pellet was resuspended into 1% (wt/vol) Sarkosyl (Sigma Chemical Co.) in 10 mM Tris-HCl volume (5). After centrifugation for 10 min at 10,000 x *g*, the OMVs were pelleted for 1 h at 50,000 x *g*. This procedure was repeated once. The final pellet was resuspended into 10 mM Tris-HCl pH 8.0; this was one-tenth of the original Tris volume. Protein concentration was measured with the BCA (bicinchoninic acid) Protein Assay Reagent (Pierce Chemical Co.) using bovine serum albumin as a standard. Desoxycholate (DOC) treatment of OMVs was carried out as follows: OMVs were resuspended into 1.5% (wt/vol) DOC, 50 mM Glycine, 5 mM EDTA pH 9.0, sonicated for 5 min, and pelleted by centrifugation for 10 min at 10,000 x *g* followed by 1 h at 100,000 x *g*. The LPS content of OMVs was approximately 25% (wt/wt LPS/protein); when treated with DOC, it was approximately 10% by KDO determination.

Characterization of OMPs. The OMPs were characterized for trypsin sensitivity and Triton X-100/mg of Cl_2 extractability as previously described (19).

Purification of antigens. PT was purified from the culture supernatant by Affigelblue (Pharmacia) column chromatography as described previously (18). PT without enzymatic activity (9K, 129G) was kindly sent by Rino Rappuoli (13). FHA was purified from the culture supernatant by Affigelblue and hydroxyapatite column chromatography. Purified 69-kDa OMP was donated by Rino Rappuoli (17). Fimbriae were isolated by heat-shock treatment of bacteria in the presence of urea, followed by some centrifugation steps (13). Endotoxin was purified by phenol extraction of bacteria (2). The 92-kDa and 32-kDa OMPs were purified from the culture cell pellet as follows. First, cells were extracted with 0.5 m $CaCl_2$, 0.14 m NaCl, 1% Zwittergent 3-14 (Calbiochem-Behring), pH 4.0 (10 ml/g wet weight of cells). After resuspension the pH was adjusted to pH 5-6. After stirring for 1 h at room temperature, the suspension was centrifuged (1 h at 3,000 x *g*); the supernatant was treated with 20% (vol/vol) ethanol, stirred for 30 min, and centrifuged for 30 min at 10,000 x *g*. The supernatant was dialyzed against 50 mM Tris-HCl, 0.05% Zwittergent 3-14, pH 8.0. After centrifugation for 30 min at 10,000 x *g*, the supernatant was brought onto a DEAE Sepharose CL6B (Pharmacia) column and eluted with a linear gradient of 0-0.6 M NaCl in 50 mM Tris-HCl, 0.05% Zwittergent 3-14. Fractions enriched in either 92- or 32-kDa OMPs were pooled, concentrated with polyethylene glycol 20,000, and precipitated with 80% ethanol. The pellets were dissolved in 50 mM Tris-HCl, 0.5% Zwittergent 3-14, pH 9.0, and run over a Sephacryl S300 column in 50 mM Tris, 0.05% Zwittergent 3-14, pH 8.0. Fractions containing 92 kDa or 32 kDa OMPs were pooled, concentrated, and stored. The 38-kDa OMP was purified from C-mode cells after OMV isolation. OMV pellets were treated with 5% Zwittergent 3-14, 50 mM Tris pH 7.5 and stirred for 2 h at 37°C. After centrifugation for 1 h at 50,000 x *g* the supernatant was run over a Sephacryl S300 column in 0.05% Zwittergent 3-14, 50 mM Tris pH 8.0. Fractions containing 38-kDa OMP were pooled, concentrated, and stored.

Electron microscopy. Electron microscopy was performed by suspending OMVs in 0.1 M ammonium acetate (pH 7.0). A droplet of this was placed on grids coated with a carbon-reinforced formvar film. After 30 s, the excess fluid was removed by absorption with filter paper and the grids stained with 2% (wt/vol) phosphotungstic acid pH 5.2 (with KOH). Examination was done with a Philips EM 400 T operated at 80 kV and at magnifications from 30,000 to 200,000.

Preparation of monoclonal antibodies. BALB/c mice were immunized intraperitoneally with 20 µg of purified antigens or OMV in the presence of 0.5 mg $AlPO_4$. ($AlPO_4$ gel was prepared from $AlCl_3$ and Na_3PO_4, equivalent quantities, pH 7.0 adjustment with 20% [wt/vol] Na_2CO_3). The mice were boosted four times at weekly intervals. After the final boost, spleen cells were fused with mouse Sp 2/0 myeloma cells as described previously (1), and the hybridoma cells cultured. After satisfactory cell growth, culture supernatants were tested by enzyme-linked immunosorbent assay (ELISA) with purifed antigens and OMVs. Interesting hybridomas were cloned twice by the limiting dilution method. From these clones, ascitis fluid was obtained after installation into the peritoneal cavities of Pristane-primed mice. Our collection of monoclonal antibodies against *B. pertussis* antigens was described previously (J. T. Poolman, B. Kuipers, M. L. Vogel, H. J. Hamstra, and J. Nagel, Microb. Pathog., in press).

ELISA. 100 µl of 2 µg/ml antigen in phosphate-buffered saline (LPS in Na_2CO_3 pH 9.0) was coated into the wells of round-bottom polyvinyl chloride microtiter plates. Purified OMPs were diluted to keep the concentration of Zwittergent 3-14 below 0.001%. PT ELISA was preceded by coating using fetuin for 16 h at room temperature. The plates were washed twice with tapwater + 0.02% Tween 80 (Merck). The reactions were carried out as described previously (1); however, 0.05% (wt/vol) Protifar (Nutricia) replaced casein to block unspecific binding. Rabbit anti-mouse peroxidase (homemade) was used as a secondary antibody. The results were measured at 450 nm using a Titertek Multiscan (Flow Laboratories, Inc.). WC ELISA was carried out as described previously (1) using Protifar.

SDS-PAGE and immunoblotting. Purified antigens, OMVs and WCVs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained as described previously (19). WCs were prepared for SDS-PAGE by suspending 3.5 ml bacteria with 1.0 absorbance at 620 nm in 70 g water + 130 µl sample buffer. Electroblotting onto nitrocellulose paper after SDS-PAGE was carried out at 140 mA constant current for 1 h on a semi-dry electroblotter (Ancos) according to manufacturer instructions. The paper was washed for 30 min with saline + 10 mM Tris pH 7.4 containing 0.5% Tween 80 at 37°C in the same buffer. The paper was washed three times with the buffer, incubated with 0.5% Protifar in buffer for 10 min washed, and incubated with rabbit anti-mouse peroxidase for 1 h in buffer + 0.5% Protifar. The paper was washed three more times and incubated with a solution containing 24 mg 3,3′,5,5′-tetramethylbenzidine (Sigma), 80 mg DONS, dioctylsulfosuccinate, dissolved in 10 ml 96% ethanol, added to 30 ml 10 mM Na_2HPO_4, 5 mM citric acid pH 5.0, to which 20 µl 30% H_2O_2 was added.

Bactericidal assay. The assay was carried out using murine monoclonal antibodies and guinea pig complement. Bacteria (10^4/ml), antibody dilution in saline, and complement were mixed in a 1:1:1 ratio using a final complement concentration of 10%. Guinea pig complement was adsorbed with agarose to remove nonspecific activity. Complement killing was allowed to proceed for 1 h at 37°C in microtiter plates, after which inoculation of Bordet-Gengou plates was carried out.

RESULTS

Table 1 shows the results of the i.c. mouse protection assay as carried out for the various preparations indicated. Besides the reference WCV preparation, several antigenic preparations demonstrated a measurable protection capacity. These preparations were PTd (either prepared by formaldehyde or site-directed mutagenesis), FHA, and OMVs. Purified antigens such as fimbriae and 69-, 92-, 38-, and 32-kDa OMPs did not reveal measurable protection capacity.

Fig. 1 shows the characteristics of the several preparations as analyzed by SDS-PAGE and electroblotting after staining with a set of monoclonal antibodies (anti-PT; -FHA; -fimbriae; and -92-kDa, -69-kDa, -38-kDa, and -32-kDa OMPs) for specific proteins or Coomassie for total protein. The Figure shows that the reference WCV preparation predominantly contains OMPs and that OMV preparations consist of 38-, 33-, and 18-kDa OMPs when grown in the avirulent phase and additional 92-, 32-, and 30-kDa OMPs when grown in the virulent phase (12).

As shown in Fig. 2, the avirulent-phase OMPs (38-, 33-, and 18-kDa) can be differentiated from the virulent-phase OMPs (92-, 32-, and 30-kDa) on the basis of trypsin sensitivity and Triton X-100/$MgCl_2$ extractabil-

TABLE 1. I.C. mouse protective units of *B.pertussis* antigens

Preparation (WCV)	Protective Units
KH 85/1 (whole cell vaccine)	6.0
PTd (formaldehyde)	12.8
PTd (9K/129G mutant)	14.9
FHA (BP-TOX6)	11.8
OMV (BP-TOX6)	6.3
OMV/DOC (BP-TOX6)	4.3
OMV (BP-TOX6. C-mode)	1.3
OMV (BP 509)	8.1
OMV (BP 509. C-mode)	2.7
OMV (BP 509. *fim*$^-$ mutant)	9.8
OMV (BP 509. FHA$^-$ mutant)	6.2
OMV (BP 134)	9.5
OMV (BP 134. C-mode)	3.1
OMV (BP W28)	3.1
OMV (BP W28. 69 kDa$^-$ mutant)	5.0
FIM 2 and 3	ND
69 kDa OMP	ND
92 kDa OMP	ND
38 kDa OMP	ND
32 kDa OMP	ND

ND, Not detectable

ity. This finding indicates that the virulence-associated OMPs are more surface exposed.

The analysis for the presence of endotoxin (*Limulus* test, *Limulus* amoebocyte) and PT (Chinese hamster ovary cell clustering, Chinese hamster ovary and lymphocytosis induction in mice, and in vivo LPF) revealed that PT activity could only be detected in WCV preparations (data not shown). The endotoxin content of FHA was high. Whole-cell and OMV vaccines contained comparable levels of endotoxin; DOC treatment of OMVs reduced the endotoxin titer by a factor of 10^4. DOC treatment caused a drop in vesicle size from 40 to 180 nm (OMV) to 10 to 25 nm (OMV-DOC) (data not shown).

Table 2 shows the serological analysis of antibodies induced in mice by a one-dose immunization with WCV, PT, FHA, OMV, fimbriae, 92K OMP, and 38K OMP. The results demonstrate that the reference WCV (from X-mode) as prepared in the liquid fermentation system does not induce detectable antibody formation after one dose against fimbriae, PT, or FHA. PTd, FHA, and fimbriae induce antibodies as detected by WC and OMV. However, the WC and OMV preparations used as a coating antigen do contain some FHA and fimbriae (see Fig. 1). On the other hand, FHA and fimbriae preparations may contain some OMPs. OMV vaccines induce some antibodies against fimbriae and FHA.

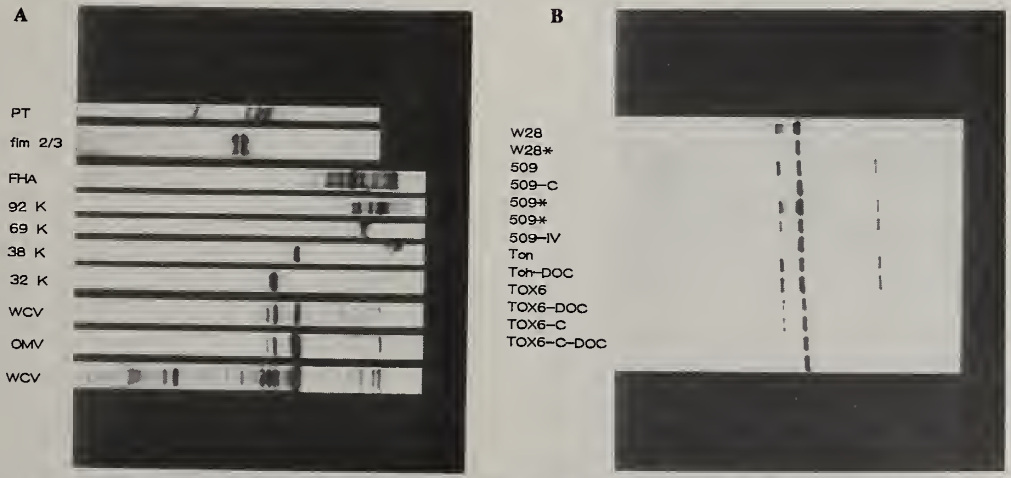

FIG. 1. (A) SDS-PAGE of *B. pertussis* WCV, OMV, and purified antigens. Lane 1, purified PT (Coomassie stain); Lane 2, purified Fim 2 and 3 (Coomassie stain); Lane 3, FHA; Lane 4, 92-kDa OMP; Lane 5, 69-kDa OMP; Lane 6, 38-kDa OMP; Lane 7, 32-kDa OMP; Lane 8, WCV; Lane 9, OMV; Lane 10, WCV (Coomassie stain). Lanes 3 to 9 were stained after electroblotting with a mixture of monoclonal antibodies against PT; FHA; Fim 2 and 3; and 92-, 69-, 38-, and 32-kDa OMPs. (B) OMP preparations stained after blotting with the monoclonal mixture. Lane 1, OMV W28; Lane 2, OMV W28 69-mutant; Lane 3, OMV 509; Lane 4, OMV 509 C-mode; Lane 5, OMV 509 FHA$^-$ mutant; Lane 6, OMV 509 *fim*$^-$ mutant; Lane 7, OMV 509 phase IV; Lane 8, OMV Tohama; Lane 9, OMV Tohama, DOC treated; Lane 10, OMV BP-TOX6; Lane 11, OMV BP-TOX6 DOC treated; Lane 12, OMV BP-TOX6 C-mode; Lane 13, OMV BP-TOX6 C-mode, DOC treated.

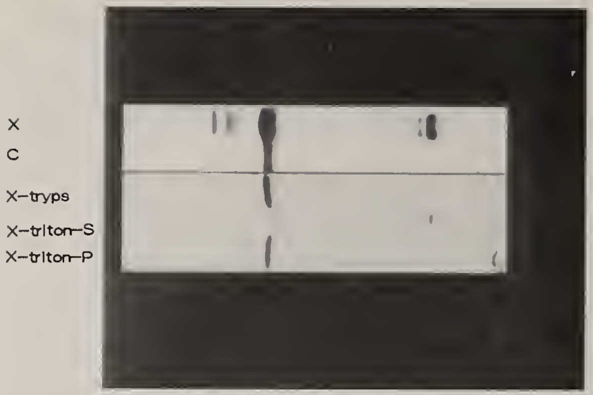

FIG. 2. OMP characterization. Lane 1, OMV Tohama, X-mode; Lane 2, OMV Tohama, C-mode; Lane 3, OMV X-mode after trypsin treatment; Lane 4, OMV treated with Triton X-100/$MgCl_2$, sup; Lane 5, OMV treated with Triton X-100/$MgCl_2$, pellet Coomassie staining.

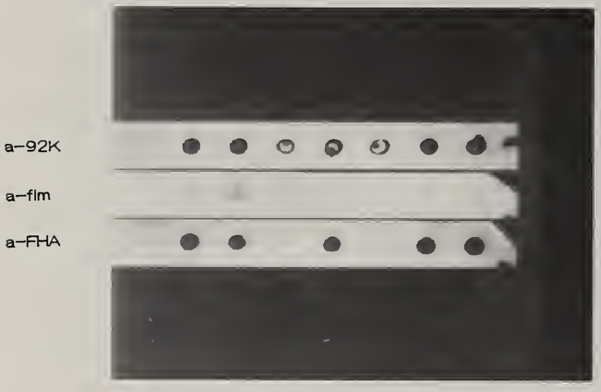

FIG. 3 Colony variants of *B. pertussis* strain 509. Lane 1, anti-92-kDa OMP; Lane 2, anti-Fim 2; Lane 3, anti-FHA.

To rule out the possibility that OMV vaccines induce protection because of antibodies against fimbriae and/or FHA, the researchers isolated fimbriae$^-$ and FHA$^-$ variants from strain 509 by colony blotting using monoclonal antibodies. Fig. 3 shows an example of colony blots and Fig. 1 demonstrates the absence of fimbriae and FHA. OMV vaccines made from such variants revealed similar protective capacities as OMV from the wild-type 509 strain. The loss of FHA caused a little reduction in mouse protection capacity. OMV vaccines were fully protective at 1-µg doses, containing not less than 1 ng PT as detected by an in vivo leukocytosis test (data not shown), which ruled out a possible effect of PT (10, 15).

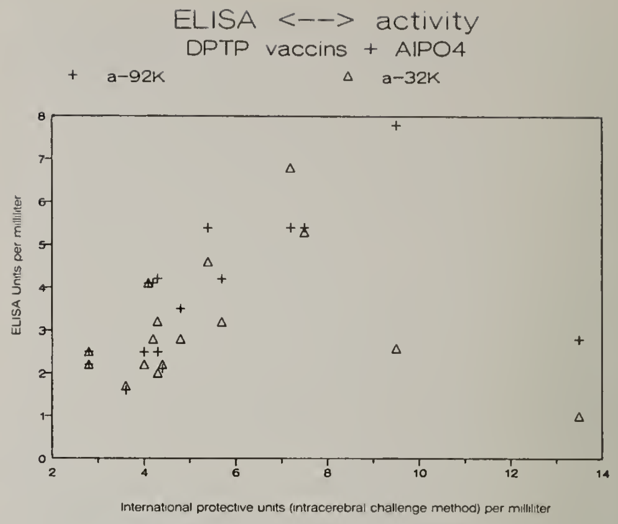

FIG. 4. Presence of 92-kDa and 32-kDa OMPs in WCV from The Netherlands versus mouse potency. ELISA units are optional density values representing binding of monoclonal antibodies to 92 kDa and 32 kDa OMPs in DPTP vaccine lots. DPTP vaccines were adsorbed on $AlPO_4$. +, anti-92 kDa, Δ, anti-32 kDa.

OMV vaccines from the PT$^-$ strain BP-TOX6 induce comparable, albeit somewhat reduced, protection as compared to strain 509. OMV from strain 134 having

TABLE 2. Antibody response in mice immunized with WCV, OMV fimbriae, FHA, PT

	ELISA-antigen						
Vaccine	WC	OMV	LPS	fim 2	fim 3	PT	FHA
WCV 12.5	1.721	2.221	0.093	0.085	0.038	0.030	0.046
PTd (formaldehyde)	0.750	0.871	0.059	0.110	0.097	2.310	0.479
PTd (mutant)	0.305	0.282	0.048	0.080	0.072	2.996	0.285
FHA	0.498	1.031	0.026	0.018	0.066	0.043	2.443
OMV	2.616	3.270	0.648	0.633	0.185	0.038	0.699
Fim 2 and 3	2.508	3.169	0.037	1.826	1.075	0.217	0.063
92 kDa	2.035	1.986	0.056	0.188	0.102	0.178	0.069
38 kDa	1.484	2.399	0.024	0.146	0.057	0.095	0.117

The WC preparation for testing in ELISA was made from shake flask cultures.

Table 3. Bactericidal activity of a collection of monoclonal antibodies against *B.pertussis* strain Tohama

Monoclonal antibody	Antigen	Isotype	Bactericidal titer
151C1	PT-S1	IgG1	<1/8
2B2	PT-S2	IgG1	<1/8
21C2	PT-S3	IgG1	<1/8
227	PT-S4	IgG1	<1/8
1-54	FHA	IgG1	<1/8
1-199	FHA	IgG1	<1/8
31E2	FHA	IgG1	<1/8
37F3	FHA	IgG1	<1/8
3D4	FHA	IgG1	1/32
22F10	FHA	IgG1	1/32
68A6	FHA	IgG1	1/32
228B7	Fim 2	IgM	1/8
81H11	Fim 3	IgG1	<1/8
133C6	92 kDa OMP	IgM	1/16
32C11	92 kDa OMP	IgG1	<1/8
14B4	92 kDa OMP	IgG2/3	<1/8
13E11	69 kDa OMP	IgM	<1/8
59BC11	38 kDa OMP	IgM	1/16
39H2	38 kDa OMP	IgM	1/16
30E5	38 kDa OMP	IgG2b	1/102,400
14B5	39 kDa OMP	IgG2/3	1/81,920
40E8	32 kDa OMP	IgG2b	<1/8
6465	18 kDa OMP	IgG1	<1/8
36G3	LPS	IgG1	1/2,048
49D9	LPS	IgG3	1/81,920
G10F8C3	LPS	IgG3	1/320,000
84B3	LPS	IgG3	1/4,096
40A1	LPS	IgG3	1/2,048

ities. OMV from a mutant lacking 69-kDa OMP from strain W28 also did not differ from the wild-type W28. W28 OMV induced lower levels of protection as compared to OMV from strains 134, 509, or Tohama. When strains BP-TOX6, 509, and 134 were grown into the C-mode, the protective capacity of OMV from these strains dropped about threefold (Table 1).

The major difference between X- and C-mode OMV preparations was the presence of 92-, 32-, and 30-kDa OMPs in X-mode preparations. Strains 509, 134, and Tohama expressed relatively more 92- and 32-kDa OMPs when standardized upon the 38-kDa OMP as compared to strains W28 and BP-TOX6. This difference was reflected in potency (Fig. 1 and Table 1).

Although a direct association between bactericidal activity of antibodies and mouse protection does not exist (2), our collection of monoclonal antibodies was analyzed for bactericidal activity (Table 3). Monoclonal antibodies against LPS and the 38-kDa porin OMP (12) revealed strong bactericidal activity. Most of the monoclonals belonged to the poorly complement-binding immunoglobulin G1 (IgG1) isotype; further studies are therefore needed.

Since the Netherlands WCV mainly induces antibodies to OMPs and the presence of the 92-, 32-, and 30-kDa OMPS greatly enhances the protective capacity of OMV vaccines, a possible correlation between the presence of the 92- and 32-kDa OMPs in DPTP vaccines and i.c. mouse protection capacity was studied. Fig. 4 shows an association between the presence of 92- and 32-kDa OMPs (standardized on number of cells) and the mouse i.c. potency test in the critical area between 4 to 8 IU of activity. A lower limit of 2 to 3 IU of potency is always present that is also reflected by OMV vaccines from C-mode organisms (Table 1).

DISCUSSION

The study results demonstrate that PTd, OMVs, and FHA are protective antigens in the i.c. mouse challenge model for potency of pertussis vaccines. In the case of FHA (made from a PT⁻ strain), an immune response against OMVs was measurable. However, OMVs do contain some FHA and no OMPs could be detected by immunoblotting in the FHA preparation. Clearly the presence of OMV within the FHA preparation was below the protective level. The endotoxin level of the FHA preparation was high, and further purification is needed to rule out an effect of endotoxin on FHA potency.

The following conclusions can be drawn regarding OMV vaccines:

(i) OMV vaccines from C-mode *B. pertussis* cells reveal a significant, low level of protection. OMVs from C-mode *B. pertussis* strain 134 (atypical LPS) behaved identically, ruling out the possibility of LPS as a protective antigen (2).

(ii) Three major OMPs (38-kDa, 33-kDa, and 18-kDa) are present within OMVs from C-mode organisms, with 38-kDa OMP being predominant. Monoclonal antibodies to 38-kDa OMP are strongly bactericidal.

(iii) OMVs made from X-mode organisms are greatly enhanced in mouse protective capacity as compared to OMVs from C-mode organisms. OMVs from fimbriae⁻ and/or FHA⁻ mutants behave similarly; loss of FHA tends to result in reduced potency. Three major OMPs (92-kDa, 32-kDa, and 30-kDa) differentiate X- and C-mode OMVs. OMV vaccines are protective when made from a PT⁻ mutant strain (BP-TOX6). BP-TOX6 and strain W28 express diminished amounts of 92- and 32-kDa OMPs; this is associated with lower potency. A W28 69-kDa⁻ mutant behaves similarly to wild-type W28.

(iv) After one dose in mice, the Netherlands WCV (fermentor culture, high-stirring) induced significant antibody responses against WC and OMV preparations only.

(v) There is a demonstrated correlation between mouse i.c. potency of batches of Dutch DPTP vaccines and the presence of 92- and 32-kDa OMPs.

(vi) DOC treatment of OMV vaccines greatly reduces the endotoxin content and results in somewhat reduced potency. Purified 92-, 69-, 38-, and 32-kDa OMPs do not induce a protective immune response.

The finding that avirulent-phase *B. pertussis* OMPs (likely candidate 38-kDa OMP) are protective antigens and that the virulent-phase OMPs (92-kDa, 32-kDa, and 30-kDa) are strong protective antigens in the i.c. mouse assay is an intriguing one not in accordance with earlier findings (15). To be protective, however, OMPs need to be present in a vesicle formulation; immunogenic purified OMPs are not protective, implying that conformational epitopes are lost during purification. Similar observations were made regarding other OMPs, e.g., meningococcal OMPs (unpublished observations). OMVs (40 to 180 nm) can be treated with DOC to reduce the endotoxin content by 10^4. The resulting vesicles (10 to 25 nm) are still protective antigens in the i.c. test, making them attractive vaccine candidates. Untreated OMVs revealed endotoxin levels comparable to WCV.

Earlier findings that a nonprotective dose of active PT enhances the i.c. mouse protection capacity of OMP preparations (mostly not present in vesicle formulation) (16) do not contradict the findings described here. However, OMV preparations can be protective without this activity. It also predicts that WCV made from a tox⁻ mutant would be protective; this was not tested in the present study.

Although there is no straightforward correlation between bactericidal activity and mouse protection, the finding that antibodies against 38-kDa OMP are bactericidal is noteworthy. Other immune mechanisms, e.g., phagocytosis, can be involved but are not well defined (2).

The findings that fimbriae⁻ and FHA⁻ mutants can easily be selected from *B. pertussis* cultures without loss of the virulence-associated OMPs is intriguing and suggests a specific regulatory mechanism. Loss of adhesins can be advantageous to a pathogenic microorganism at certain stages of the disease process.

It is likely that the immune mechanisms involving *B. pertussis* OMPs differ from those involving the two widely accepted antigens for inclusion in an acellular pertussis vaccine, PTd and FHA (3, 7, 11, 16). In this respect, further studies on *B. pertussis* OMPs are warranted, since the results from a field trial with a combined PTd-FHA vaccine are not totally satisfactory (3).

ACKNOWLEDGMENTS

Rino Rappuoli (Sclavo Research Center) has helped us greatly by sending BP-TOX6, purified 69-kDa OMP, and purified PT_x (9K, 129G) (13). Ineke v.Straaten-v.d. Kappelle carried out the bactericidal test; Danny Schijf-Evers helped with ELISA and column chromatography; Cyril Roosenboom carried out image processing; Marina Burger performed electron microscopy analysis; Dominique Borsboom carried out the in vivo leukocytosis test and Chinese hamster ovary clustering assay; and Elly Verhagen supplied us with *B. pertussis* fermentor cultures.

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DISCUSSION

DR. FINN: I really do not have a question, just something to add to what you were saying about the 92 kDa protein. We have, in John Mekalanos' laboratory, made some Tn4 insertion mutants and one of them is in a *vir* regulated gene which is, I think, identical to your 92 kDa protein and this strain is reduced in its ability to persist in the mouse lung in the mouse colonization lung model.

DR. POOLMAN: Are you sure that it is only the 92 kDa protein that is missing there?

DR. FINN: Well, I am presuming it is the same protein as the one you are talking about. It is approximately 90 kDa and is *vir* regulated and it is only one protein in this particular strain.

DR. ARCINIEGA: Did you test for the presence of PT and FHA in your Sarcosyl extract using monoclonal antibodies?

DR. POOLMAN: Yes.

DR. ARCINIEGA: Are they present?

DR. POOLMAN: After a couple of Sarcosyl extractions, we cannot detect them.

DR. COWELL: If that is true then, I thought I saw on your slide that you injected mice with the outer membrane vesicles and if I recall, you measured antibody and I saw antibody to FHA and fimbriae and LOS from mice being immunized with the outer membrane vesicles. So maybe you could not detect them but they must have been there.

DR. POOLMAN: Yes, there are low levels present. That is true. We cannot detect them antigenically but they do give rise to low levels of antibodies.

DR. COWELL: That one slide where you have protection with purified 92 kDa and 38 kDa with and without PT, I did not quite understand what the numbers were in the slash. They were the survivors at different times? So you were getting some protection early when you added PT?

DR. POOLMAN: It was the survivors after standard time. So you have a four-fold dilution range and then the survivors are given after the standard procedure.

DR. COWELL: I presume from the end of your talk, that the 38 kDa and the 92 kDa were two separate proteins but when you showed a gel of that, is it possible that this is the 40 kDa that Mike Brennan just talked about?

DR. POOLMAN: The 38 kDa is the same as the 40 kDa of course. That is the porin protein.

DR. COWELL: Yes, but it is heat modifiable so depending on how you heat it and do the gel --

DR. POOLMAN: Yes, but these gels are all done at 100 degrees and these proteins are clearly separate. We have lots of monoclonal antibodies that are specific for either one of them.

SESSION THREE

Immunology and Host-Parasite Interactions of *Bordetella pertussis*

Co-chairpersons:

M. Teresa De Magistris
Roberta D. Shahin

Introduction

M. TERESA DE MAGISTRIS

This session will deal with immunology and host parasite interactions of *Bordetella pertussis*. I would like to show you a simple scheme to remind you what the network between T cells, B cells and macrophages is during immune recognition.

When T lymphocytes recognize the antigen and antigen presenting cells such as B lymphocytes, macrophages in association with class two molecules on the surface of these antigen presenting cells, they get activated and produce some lymphokines such as interferon gamma, interleukin-II, interleukin-IV and other lymphokines. They can also get activated in the sense that they acquire cytotoxic activity against antigen-presenting cells. Another very important function of T cells is that of helping B lymphocytes to become plasma cells and produce antibodies against the antigen.

The T lymphocytes can modulate the immune response by means of the lymphokines they produce. In fact, for instance, interferon gamma can act on macrophages. They will get activated and will acquire the capability to kill intracellular bacteria. On the other hand, interleukin-IV can promote the production of IgE isotype antibodies and so these factors can modulate the immune response.

Today we will have the first three presentations on cell mediated immunity against *Bordetella pertussis* and then we will have two presentations on the IgE response following immunization and then there will be a presentation on mucosal immunity which is also very important for a bacterium such as *Bordetella pertussis* which colonizes mucosa and finally we will have two presentations on adenylate cyclase which is an antigen which interferes with the immune system by acting on macrophages and other cells.

Humoral and Cellular Immunity in Adult Volunteers Immunized with a Genetically Inactivated Mutant of Pertussis Toxin

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The cellular vaccine against whooping cough, although efficacious, is reactogenic and has been associated with some rare but severe complications, such as neurological damage and death (5). Acellular vaccines containing chemically detoxified pertussis toxin (PT) have fewer side effects and are efficacious in preventing severe disease (1,8). However, chemical detoxification can alter the structural characteristics of PT, reducing its immunogenicity and, in some cases, allowing reversion to toxicity (11).

To avoid these consequences in the present study, the gene coding for PT was modified and strains of *Bordetella pertussis* were obtained that produce modified PT devoid of enzymatic activity (9). Such molecules retain the natural epitopes of PT which may be lost during chemical detoxification. In addition, they retain other properties of PT such as the ability to bind receptors on eukaryotic cells and be mitogenic for T cells which are not present in chemically detoxified molecules (6). When tested in animal models, genetically detoxified PT was confirmed (i) to be nontoxic and immunogenic and (ii) to protect mice from intracerebral challenge with virulent *B. pertussis*. This paper reports on the safety and immunogenicity in human adult volunteers of one such PT mutant, PT-9K/129G (7, 9). The phase-I trial was designed to determine the safety of this unique molecule which, at high doses, is mitogenic for T cells in vitro. Thus, a large panel of leukocyte surface markers representing resting and activated stages of cellular subpopulations were monitored in donors' peripheral blood mononuclear cells. In addition to humoral immunity, which is usually tested in clinical trials, the cellular immune response, which is thought to be the in vitro correlate of in vivo immunological memory, was also studied. Cellular immune response is an important requirement for a candidate protein to become a new vaccine (2, 3).

MATERIALS AND METHODS

Twenty-nine healthy adult volunteers of both sexes, 25 to 58 years of age, with low anti-PT antibody titer (<20 EU/ml), were enrolled in the double-blind study and randomly assigned to the vaccine or placebo group. Eighteen volunteers received intramuscularly 0.5 ml of vaccine containing 15 mcg of PT-9K/129G, 0.05 mg of thimerosal, and 0.5 mg of aluminum hydroxide. Eleven volunteers received 0.5 ml of placebo containing 0.05 mg of thimerosal and 0.5 mg of aluminum hydroxide. After 6 weeks, 17 vaccinees and 9 placebo recipients were given a second dose of vaccine or placebo, respectively. Blood samples for counts of red and white cells and platelets as well as for levels of hemoglobin, hematocrit, glucose, urea, creatinine, SGOT, SGPT, gamma GT, immunoglobulin (IgE), and insulin (fasting values) were obtained before each injection and after 3 days (also after 1 month for IgE and insulin only). Leukocytes were analyzed for surface markers by cytofluorimetry. Titers of serum antibodies against PT before and after immunization were measured by enzyme-linked immunosorbent assay (6) and by a toxin neutralization test on Chinese hamster ovary cells (6). In vitro proliferation of leukocytes and T-cell clones in response to PT and peptides was assessed by 3H-thymidine incorporation (3).

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* Presenting author.

RESULTS AND DISCUSSION

Phase-I clinical trial with the PT mutant PT-9K/129G. A complete absence of typical adverse reactions either local or systemic was observed after the first dose of vaccine or placebo. After the second dose, 2 out of 17 vaccinees had mild local pain, erytheme, and induration; only 1 out of 9 volunteers receiving placebo had local pain. Hematological, clinical chemistry insulin, and IgE values did not show any significant variation. No difference in leukocyte numbers or population proportions was observed between the two experimental groups.

To assess the potential effect of PT-9K/129G mitogenicity (6), several leukocyte markers were analyzed 4 and 30 days after the first vaccine administration. The proportion of total T cells (CD3), T helper (CD3/CD4) and T cytotoxic (CD3/CD8) subpopulations and that of B cells (CD19), monocyte-macrophages (CD14) and NK cells (CD57) did not change after vaccination. Similarly, no changes were observed in leukocyte activation markers such as CD25 and CD23 or in the proportion of T cells bearing the gamma-delta receptor, i.e., the still largely unknown population often associated with bacterial infections (4). It was concluded that the administration of PT- 9K/129G, which at high doses is mitogenic for T cells in vitro, does not cause in vivo nonspecific activation of leukocytes.

After the first dose, a great increase in antibody titers against PT was observed in both enzyme-linked immunosorbent assay and Chinese hamster ovary cell neutralization assays (Table 1). The second dose did not cause any further increase of humoral responses, indicating that all adult volunteers had been previously exposed to *B. pertussis* and therefore gave a secondary response to the first immunization. The titers of PT-specific antibodies were higher than those reported in similar studies using higher doses of chemically detoxified PT (10). In particular, the ratio between toxin-neutralizing titers and total anti-PT enzyme-linked immunosorbent assay titers was the highest reported so far, suggesting that immunization with a molecule not chemically modified may induce antibodies with higher affinity for the native PT.

To assess whether PT-9K/129G vaccine would also increase cellular immunity, selected volunteers (two from the placebo group and six from the vaccine group) were analyzed for their in vitro proliferative response to mutant and wild-type PT (3). Table 2 shows that peripheral blood mononuclear cells are able to recognize both wild-type and mutant PT after vaccination, whereas no statistically significant increase in proliferation was observed with a nonrelated antigen such as tetanus toxoid. It was concluded that PT- 9K/129G is safe and capable of inducing both humoral and cellular responses against PT.

TABLE 1. Serum antibody responses in adult volunteers receiving placebo or PT-9K/129G vaccine

	Time	Experimental Groups			
		Placebo		Vaccine	
	(Day)	Geometric mean (95% confidence interval)	Stimulation index (vs. day 0)	Geometric mean (95% confidence interval)	Stimulation index (vs. day 0)
Neutralizing titer[a]	0	12.8 (7.5 - 21.6)		13.6 (8.6 - 21.5)	
	+30	12.8 (6.7 - 24.9)	1.0	1,810.2 (737.0 - 4,446.0)[b]	133
	+70	11.7 (5.9 - 23.0)	0.9	1,974.0 (820.1 - 4,751)[b]	145
Antibody titer[c]	0	6.5 (3.5 - 12.2)		6.7 (4.6 - 9.8)	
	+30	8.1 (4.2 - 15.5)	1.2	496.5 (199.9 - 1,233)[b]	74
	+70	7.9 (4.5 - 13.9)	1.2	522.4 (216.8 - 1,258.6)[b]	78

[a] Units in Chinese hamster ovary cell assay.

[b] $P \leq 0.01$ versus day 0 control.

[c] Enzyme-linked immunosorbent assay units based on total antibodies against PT.

TABLE 2. In vitro proliferative responses of peripheral blood monoclear cells of adult volunteers receiving placebo or PT-9K/129G vaccine

			In vitro antigen		
Experimental Group	Time (Day)	Medium	PT (1 μg/ml)	9K/129G (1 μg/ml)	Tetanus toxoid (1 μg/ml)
Placebo	0	226[a]	401 (1.8)[b]	461 (2.0)	12,043 (53.3)
	+30	376	793 (2.1)	1,496 (4.0)	14,230 (37.8)
	+84	315	847 (2.7)	1,762 (4.7)	ND
Vaccine	0	235	909 (3.9)	702 (3.0)	6,820 (29.0)
	+30	339	11,483[c] (33.9)	8,638[c] (25.5)	8,188 (24.1)
	+84	569	25,706[c] (45.2)	13,299[c] (23.4)	ND

[a] Mean of incorporated count per minute (5). Standard error was always less than 10%.
[b] Amounts given in parentheses indicate stimulation index versus control in medium alone.
[c] $P \leq 0.01$ versus day 0 control.
ND, Not done.

The safety and immunogenicity of PT- 9K/129G are at least as good as those of chemically detoxified PT molecules. Since the efficacy of PT in preventing severe whooping cough has already been shown in the Swedish clinical trial (1, 8), the presence of a molecule which eliminates all problems associated with chemical detoxification suggests that PT-9K/129G should be the antigen of choice for future acellular vaccines against whooping cough. In addition to the normal parameters usually studied in vaccine clinical trials, the present research has also addressed the cellular immune response to the antigen. Studies of this response might be useful in the future to evaluate vaccine potency and avoid extensive clinical trials, especially when these trials require numbers which are logistically difficult to reach.

Immunization with PT-9K/129G generates T cell response against the immunodominant epitope of the S1 subunit. It has been shown previously that adult volunteers who had acquired immunity to pertussis from the disease have a T cell immunity directed against an immunodominant epitope containing amino acids 30-41 of the S1 subunit of PT (3, 7; De Magistris, manuscript in preparation). To investigate whether immunization with PT-9K/129G generates immunity against the same epitope, peripheral blood cells from volunteers immunized with two doses of PT-9K/129G were stimulated with a synthetic peptide covering amino acids 30-42 of the S1 subunit. T cell clones specific for the 30-42 peptide were isolated from three donors with different HLA genotypes and tested for recognition of PT, PT-9K/129G, and several peptides overlapping the 30-42 region. All clones were able to recognize PT and PT-9K/129G but had a different fine specificity towards the peptides (Table 3). Therefore, T cell immunity against the immunodominant epitope 30-42 can be induced ei-

TABLE 3. Fine specificity of human T cell clones obtained from volunteers with different HLA genotype.

Clone	Antigen						Donor HLA-DR
	PT	PT9K/ 129G	30-42	27-40	27-39	30-39	
EB-2	+	+	+	+	+	+	1,2
GS-1	+	+	+	–	–	–	4,w8
ML-4	+	+	+	+	–	–	2,9

TABLE 4. Effect of alanine substitutions on T cell recognition of peptide 30-42

	Ala-substituted residue												
	30	31	32	33	34	35	36	37	38	39	40	41	42
Clone	D	N	V	L	D	H	L	T	G	R	S	C	Q
S106	+	++	++	+	−	−	−	+	+	+	+	+	+
S213	+	++	+	+	−	−	−	−	+	−	−	+	+
S2332	+	+	+	+	−	+	−	−	+	−	ND	+	+
T218	+	+	+	+	−	−	+	−	+	−	+	+	+
T220	+	++	+	+	−	−	+	−	+	−	+	+	+
T219	+	+	+	+	−	+	+	−	+	−	++	+	+
S208	+	++	++	+	+	+	+	−	+	−	+	+	+
T215	+	+	+	+	−	−	−	−	+	+	++	+	+
EB-2	+	+	+	+	−	−	−	−	+	−	++	+	+
GS-1	+	+	++	+	−	−	+	−	+	−	++	+	+
ML-4	+	+	++	+	++	+	+	−	+	−	+	++	+

ND, Not determined

ther by vaccination with the mutant PT-9K/129G or by the disease.

Identification of the amino acid residues of peptide 30-42 involved in T cell receptor binding. To better understand the properties of the epitope 30-42, 13 analogs of peptide 30-42 were synthesized, each containing a different Alanine substitution. These mutated peptides were then tested for their capacity to stimulate the proliferation of the T cell clones obtained either from a donor who had disease (2) or from the vaccinated volunteers described above. The results showed that (i) although all T cell clones are specific for peptide 30-42, they have a different fine specificity for the peptide analogs; (ii) Alanine substitution in positions 34, 35, 36, 37, and 39 abolishes T cell recognition in most of the clones; and (iii) in some cases, Alanine substitution in positions 31, 32, and 40 increases the stimulatory potency of the analogs (Table 4).

Taken together, these results show that the same epitope is recognized by T cell clones with different T cell receptors, each recognizing the peptide in a slightly different way. However, most of the clones require a core of five amino acids for recognition. It cannot be determined whether these five amino acids are all required for T cell receptor binding or whether some of them are also important for binding to HLA.

Interestingly, some of the substitutions increase the stimulatory potency of the peptides. This finding suggests that, in theory, the immunogenicity of the 30-42 epitope can still be improved. Some of these mutations are now being introduced in the PT-9K/129G to verify whether they improve the epitope when included in the entire protein. Engineering of T cell epitopes to improve the potency and immunogenicity of vaccine molecules is likely to be one of the major trends in future vaccinology.

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DISCUSSION

DR. HUANG: I did not hear that you had separated the lymphocytes into T cells and B cells so how do you know those mitogenic activity was all because of T-cells? The second thing is I do not think that we could equate the mitogenic activity with the T cell immunity. You probably had to do some more effector type of study like migration inhibitory factor or did you do any skin test that verified that the patient had a delayed-type hypersensitivity?

DR. DE MAGISTRIS: We did not separate the cells.

DR. HUANG: So how do you know that you are dealing with T cells? Are you sure the B cell did not proliferate?

DR. DE MAGISTRIS: What we measured was not a specific mitogenic effect because we heat the denatured toxin before we put it into the test. Since lymphocytes can see the linear epitopes on the antigen-presenting cell by eliminating the mitogenic effect of pertussis toxin with heat inactivation, we know the we are looking only at the T-cell response and not the B-cell response.

DR. HUANG: The second question is that I do not think it is fair to say the mitogenic response is equal to T-cell immunity. I would like to see some more effector type of response of the T-cell like a delayed-type hypersensitivity test. Did you have a chance to do skin tests on those patients?

DR. DE MAGISTRIS: We did not do skin tests.

DR. SHAHIN: Your cytotoxic T-cell clones, were they class one restricted or class two restricted?

DR. DE MAGISTRIS: They were class two restricted. They were all CD-4 positive T-cell clones and recognized the antigen. They exerted killing activity only when the antigen was presented by autologous antigen presenting cells or matched for the class two molecules; so they are CD-4 positive.

DR. ARCINIEGA: From an academic point of view, have you tried to address or to answer the question of whether the B-oligomer is mitogenic at short distances from the point of inoculation?

DR. DE MAGISTRIS: If you mean in vivo, we did not try.

PARTICIPANT: I am wondering, is there any evidence that after infection of *Bordetella pertussis* in macrophages, these pertussis toxin epitopes get exposed?

DR. DE MAGISTRIS: On macrophages? No, we did not use macrophages. We always used B cells as antigen presenting cells but I am sure that if we gave the antigen to autologous macrophages, they would present the antigen as well as B cells.

PARTICIPANT: You did not test that?

DR. DE MAGISTRIS: We did not test it with purified macrophages but when we tested the proliferation of peripheral blood lymphocytes to the peptides or to the entire protein, then that is what happens. The macrophages present the antigen to the T cells and then we measure proliferation.

MR. PREBULA: In your in vivo experimentation, you measured proliferation of T cells by using anti-CD25 antibodies and then doing FACS analysis. Did you consider measuring soluble IL-2 receptor, the P-55?

DR. DE MAGISTRIS: The IL-2 receptor? No, we only performed FACS analysis on the cells, not on the soluble antigen.

Specificity of the T-Cell Response to *Bordetella pertussis* in Aerosol-Infected Mice

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The need for a safe and effective acellular pertussis vaccine requires a full understanding of the mechanism of the protective immune response as well as the identification of immunologically active *Bordetella pertussis* components for inclusion in a subunit or synthetic vaccine. Several *B. pertussis* antigens have been examined either alone or in combination as potential vaccines, and murine models have been used to study efficacy in relation to the serological responses generated (9, 15, 19, 21). Active or passive immunization with detoxified pertussis toxin (PTd) has been shown to protect mice against intracerebral and respiratory challenge (15, 19). The filamentous hemagglutinin (FHA) and the 69-kDa outer membrane protein (OMP) have also been shown to have a protective role; however, their efficacy as monovalent vaccines is more controversial (9, 13, 15, 18, 21). Clinical trials with an acellular vaccine have shown that purified inactivated pertussis toxin (PT), either alone or with FHA, can confer protection but not to the same level as that achieved with the whole-cell vaccine (1, 13). Furthermore, a lack of correlation between serological responses to PT and protection has been reported (1, 13), suggesting that alternative arms of the immune response and/or other antigens may be involved in protective immunity to *B. pertussis.*

Although neutralizing antibodies are considered to be the major protective mechanism against infection with *B. pertussis* (15, 18, 19), the role of cell-mediated immunity has been poorly addressed. In addition to providing helper function for B-cell production of antibody, antigen activated T-helper (Th) cells secrete a variety of cytokines which stimulate macrophages and granulocytes or mediate direct activity on foreign microorganisms (11). Furthermore, cytotoxic T cells have been shown to play a role in the control of diseases caused by certain bacteria, particularly by intracellular pathogens such as *Mycobacteria* spp. (7).

The murine aerosol infection model has been used in an analysis of the mechanism of protective immunity to *B. pertussis.* This model is considered by many to be more satisfactory than the murine intracerebral challenge model in that many of the parameters of infection are similar to that observed in infants (9, 18, 20). It was shown that transfer of immune T cells from mice that had recovered from a respiratory pertussis infection into irradiated immunosuppressed mice or T-cell-deficient nude mice resulted in rapid clearance of bacteria from the lungs following challenge (K. H. G. Mills, A. Barnard, S. Watkins, and K. Redhead, manuscript in preparation). The present study on the antigen specificity of T cells from the immune animals revealed that CD4$^+$ memory T cells directed against FHA, the 69-kDa OMP, PT, and agglutinogens 2 and 3 (Aggs 2+3) were generated following a sublethal respiratory infection.

MATERIALS AND METHODS

Mice. BALB/c mice were bred and housed at the National Institute for Biological Standards and Control, Hertfordshire, United Kingdom. Male and female mice 3 to 4 months old were used for aerosol infection.

Aerosol infection. Lung infections were initiated using a method similar to that described by Sato et al (20). Mice were exposed to aerosols of *B. pertussis* Wellcome 28 phase 1, generated using suspensions containing approximately 2 x 10^{10} bacteria/ml. Progress of the infection was followed by viable counts of bacteria. Mice were killed by cervical dislocation, the lungs were removed aseptically, and homogenized in sterile physiological saline containing 1% (wt/vol) casein, and the number of CFUs of *B. pertussis* were determined on Bordet-Gengou plates.

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* Presenting author.

Antigen preparations for T cell assays. Whole *B. pertussis* Wellcome 28, was heat inactivated at 60°C for 30 min and used at 10^5 to 10^9 bacteria per ml in culture. Supernatant from sonicated *B. pertussis* was inactivated by treatment with 0.4% of formalin followed by exhaustive dialysis against phosphate-buffered saline. Purified native PT, FHA, 69-kDa OMP, and detoxified versions of the same antigens prepared by treatment with gluteraldehyde followed by formalin (PT) or formalin alone (FHA and 69-kDa OMP) were kindly provided by Carine Capiau at SmithKline Biologicals, Rixensart, Belgium. Alternative preparations of FHA and PTd (designated FHA-1 and PTd-1 respectively) were purified or detoxified at the National Institute for Biological Standards and Control. The FHA-1 was purified by the method of Robinson et al. (17), and the PTd-1 was detoxified with formalin followed by dialysis against phosphate-buffered saline. Recombinant 69-kDa OMP expressed and purified from *E. coli* was kindly provided by Gordon Dougan, Ian Charles, and Neil Fairweather at Wellcome Biotechnology, Beckenham, United Kingdom. Purified Aggs 2+3 was provided by Andrew Robinson at CAMR, Wiltshire, United Kingdom. Lipopolysaccharide (LPS) was prepared from *B. pertussis* Wellcome 28 by the method of Westphal and Jann (23). Protein concentrations of antigen preparations were estimated by Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.).

Proliferation assay. Spleen cell suspensions were prepared as previously described (22) and suspended in RPMI medium supplemented with 50 µm of 2-mercaptoethanol and 8% heat-inactivated fetal calf serum or 2% normal (BALB/c) mouse serum. The spleen cells were cultured at 2 x 10^6/ml in 200-µl volumes in triplicate wells of 96-well microtiter plates with various antigens in the dose range of 8 ng to 25 µg per ml. Unless otherwise indicated, cultures were incubated for 4 days at 37°C in a humidified CO_2 incubator. Proliferation was assessed by [^{3}H]thymidine incorporation by adding 0.5 µCi of [^{3}H]thymidine per well 4 h before the end of the culture period. The cells were harvested using an automated cell harvester and [^{3}H]thymidine incorporation was measured by scintillation counting. Results were expressed as mean counts per minute for triplicate cultures.

IL-2/IL-4 assay. Lymphokine levels were measured by the ability of the supernatant to support the growth of the IL-2/IL-4 dependent CTLL-2 cell line. The CTLL-2 cell line was shown to proliferate in response to recombinant IL-2 or IL-4; these responses were inhibited by monoclonal antibodies to the IL-2 receptor or IL-4, respectively. Spleen cells were cultured in triplicate as described above for the proliferation assay. Supernatants were removed after 24 h of culture and stored at -20°C. Next, 50 µl of supernatant and 50 µl of CTLL cells (1 x 10^4) were added in triplicate to wells of flat-bottomed microtiter plates. Growth of the CTLL line was measured by [^{3}H]thymidine incorporation assessed after 24 h of culture. The results were expressed in counts per minute for triplicate assays.

RESULTS

Certain *B. pertussis* components, such as PT and LPS, are known to have mitogenic activity for murine T and B lymphocytes, respectively. Consequently, the use of proliferation assays to assess T-cell immunity to *B. pertussis* is complicated by high background activity observed in unseparated lymphocyte populations in response to purified antigens contaminated with even low levels of PT or LPS. Therefore, the release of lymphokines IL-2 and IL-4 mainly attributed to CD4$^+$ Th cells (14) were examined as a more specific assay of T-cell responses. (Although the predominant lymphokine detected was IL-2, the lymphokine is herein referred to as IL-2/IL-4.) A comparison of proliferation and IL-2/IL-4 release by spleen cells from normal nonimmune mice was made (Fig. 1). The high mitogenic activity of the heat-treated whole *B. pertussis* bacteria, a formalin-treated *B. pertussis* sonicate, purified active PT, and LPS was reflected in the strong proliferative responses observed with these preparations. In addition, one of the two FHA preparations (FHA-1), and the Aggs 2+3 preparation also induced significant lymphocyte proliferation at the highest dose tested (5 µg/ml), presumably reflecting a high level of contaminating PT or LPS. In contrast, when lymphokine release was assessed after stimulation of normal lymphocytes with the same antigen preparations, only active PT induced a significant response and then only at the higher concentrations tested (1 to 5 µg/ml) (Fig. 1 and 2). The heat-treated whole *B. pertussis* organism and the FHA-1 preparation also stimulated low levels of lymphokine release in one of four animals tested. Therefore, apart from the active PT preparation used at high concentration, all of the *B. pertussis* antigen preparations could be used for testing antigen-specific T-cell responses in immune animals.

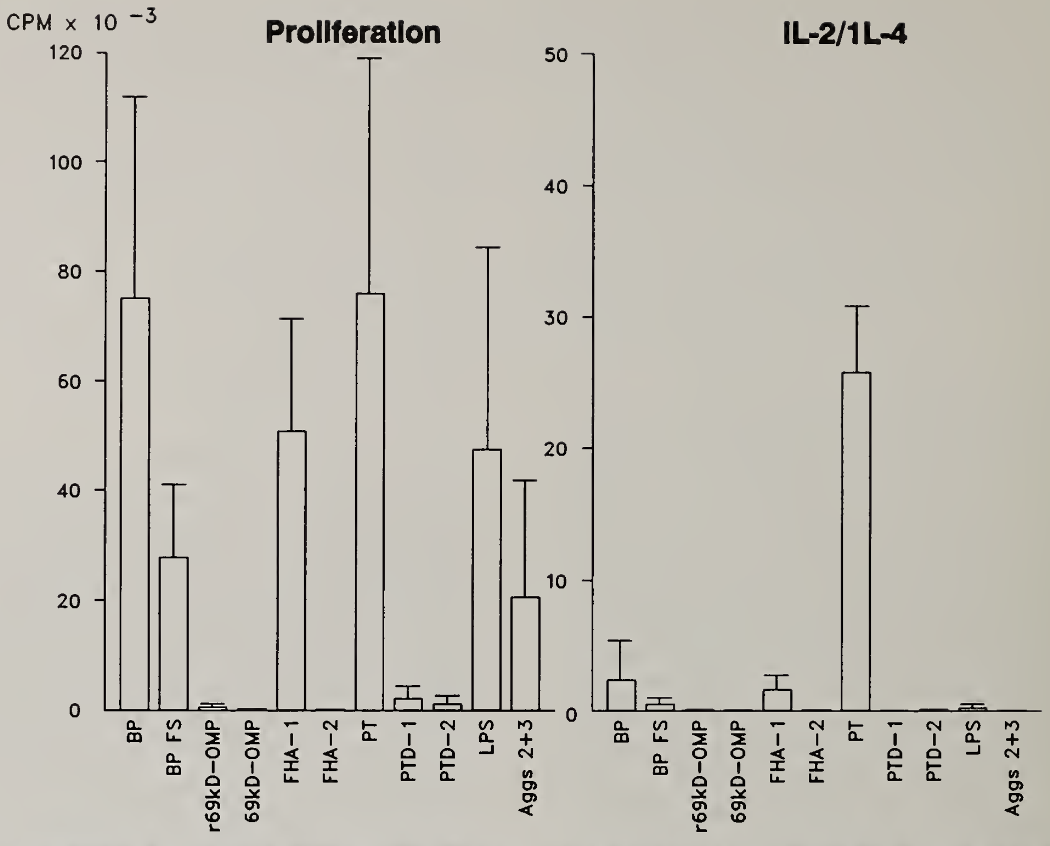

FIG. 1. Proliferation and lymphokine release by spleen cells from normal nonimmune mice to *B. pertussis* antigens. The results represent mean ± SD responses from four animals with the optimum concentration of heat-treated *B. pertussis* (BP) in the range of 10^5 to 10^9 bacteria per ml and the following soluble antigen preparations in the range of 0.04 to 5 μg/ml: formalin-treated *B. pertussis* sonicate (BP FS), recombinant 69-kDa OMP (r69kDa-OMP), purified native 69-kDa (69kDa-OMP), FHA prepared at the National Institute for Biological Standards and Control (FHA-1), or provided by SmithKline (FHA-2) active PT, formalin-inactivated PT (PTd-1), glutaraldehyde- and formalin-inactivated PT prepared by SmithKline (PTd-2), LPS, and Aggs 2 + 3.

In three separate experiments, spleen cells from six immune mice and four nonimmune control mice were tested for lymphokine release in response to *B. pertussis* antigen preparations (Fig. 1). Mice that had been aerosol challenged 6 to 12 weeks earlier with *B. pertussis* and had cleared the bacteria from their lungs were used as a source of immune spleen cells. The total bacterial count in the lungs upon challenge was approximately 10^6. This count increased to a peak of 10^7 to 10^8 by day 7; the bacteria were completely cleared by approximately 35 days post challenge. The lymphokine response to a range of concentrations of *B. pertussis* components is shown for individual mice (Fig. 2.) Both the whole heat-treated organism and the formalin-treated sonicate induced strong lymphokine responses in lymphocytes from each of the six immune animals tested. The responses reached a plateau at 10^6 bacteria/ml or 1 μg/ml of sonicate. High concentrations of the whole heat-treated organisms (10^9/ml) were inhibitory, suggesting a toxic activity for murine lymphocytes at this level.

Each of the individual purified *B. pertussis* components (FHA, Aggs 2+3, PT, and 69-kDa OMP) stimulated lymphokine secretion by immune T cells (Fig. 2). In terms of the concentration of antigen required to induce a significant response, FHA was the most active: in most cases, its dose-response curves reached a plateau at the lowest concentration tested (40 ng/ml). Both the purified native and recombinant 69-kDa OMP preparations induced strong responses in the range 0.2 to 5 μg/ml in lymphocytes from each of the immune animals tested. The Aggs 2+3 was only recognized by T cells from four out of five of the immune animals tested, but nevertheless stimulated high levels of lymphokine release at 1 to 5 μg/ml. Surprisingly, LPS induced a detectable lympho-

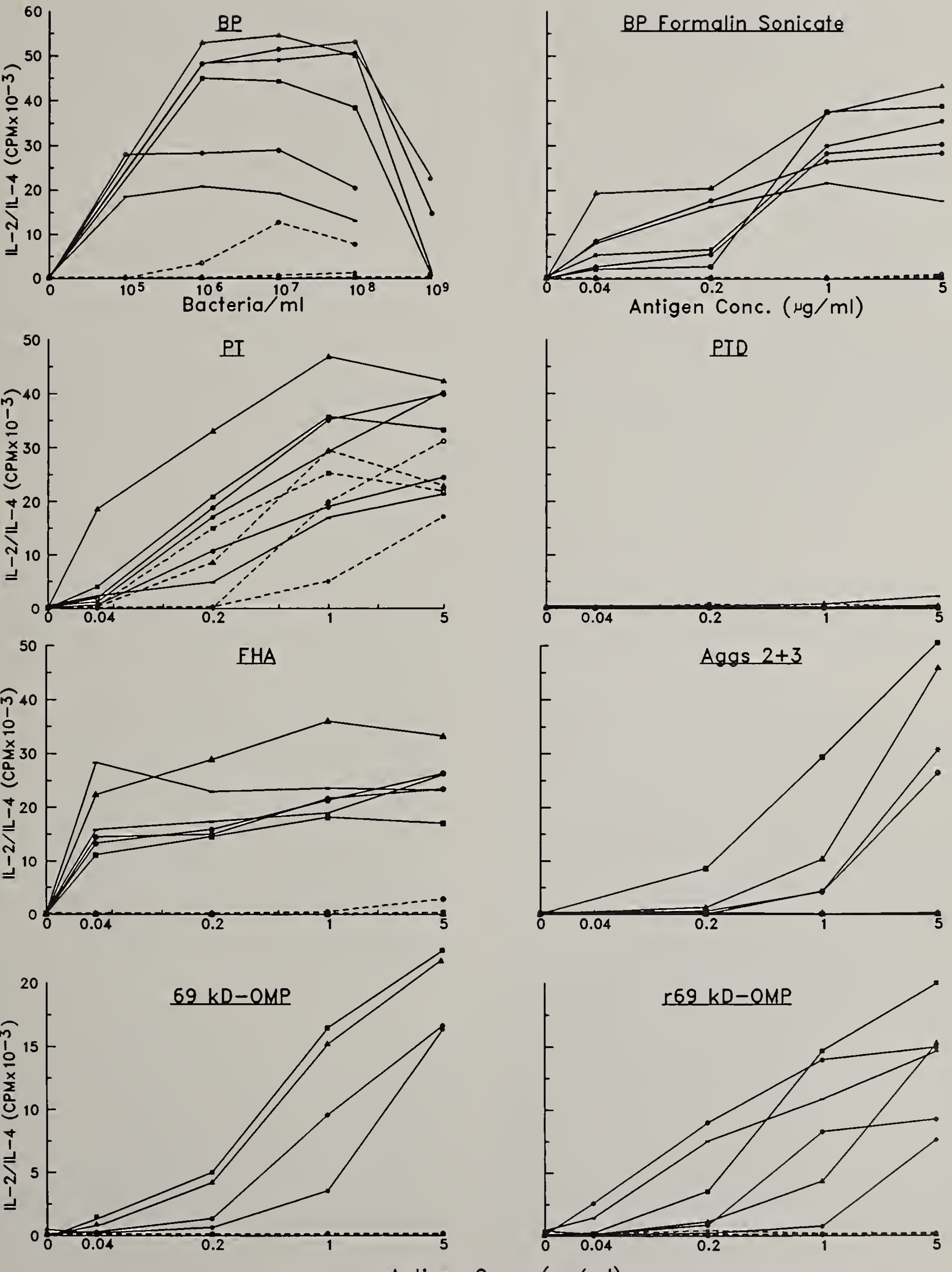

FIG. 2. Lymphokine release by T cells from aerosol-infected mice in response to in vitro stimulation with *B. pertussis* antigens. The response of six individual immune (—) and four control nonimmune (----) animals are shown against a range of doses of each antigen preparation described in Fig. 1. The lymphocyte response to native 69-kDa OMP and Aggs 2+3 was tested in only four and five immune animals, respectively.

kine response in lymphocytes for three of the six immune animals at the highest dose tested (5 µg/ml), whereas the response of four control animals was completely negative (data not shown).

Lymphocytes from the nonimmune control animals proliferated and released IL-2/IL-4 upon stimulation with active PT in the concentration range of 1.0 to 5 µg/ml (Fig. 2 and 3). However, at 0.04 µg/ml in three experiments, and at 0.2 µg/ml in two experiments, PT did not induce a significant response with the control cells. In contrast, lymphocytes from immune animals proliferated and released IL-2/IL-4 in response to 0.04 µg/ml of PT in all experiments. Furthermore, the dose-response curves in the range 0.04 to 1 µg/ml revealed significantly greater responses with immune compared with control lymphocytes in each of three experiments, regardless of the culture period (2 to 4 days) of the proliferation assay (Fig. 3). When PTd was used as the stimulating antigen, significant proliferation or lymphokine release could not be detected in control or murine lymphocytes in the concentration range of 0.04 to 5 µg/ml (Fig. 2 and Table 1).

The possibility that the lymphocyte nonresponsiveness to the PTd had resulted from a nonspecific toxicity effect of the preparations, such as residual glutaraldehyde from the detoxification process, was examined. The PTd was added to cultures of a T-cell clone 13.10 specific for influenza hemagglutinin (12) in the range of 0.04 to 5 µg/ml and was found not to affect the antigen-specific response. Furthermore, heat-treated PT (80°C, 30 min) also failed to induce an in vitro response in lymphocytes from *B. pertussis* immune or control mice

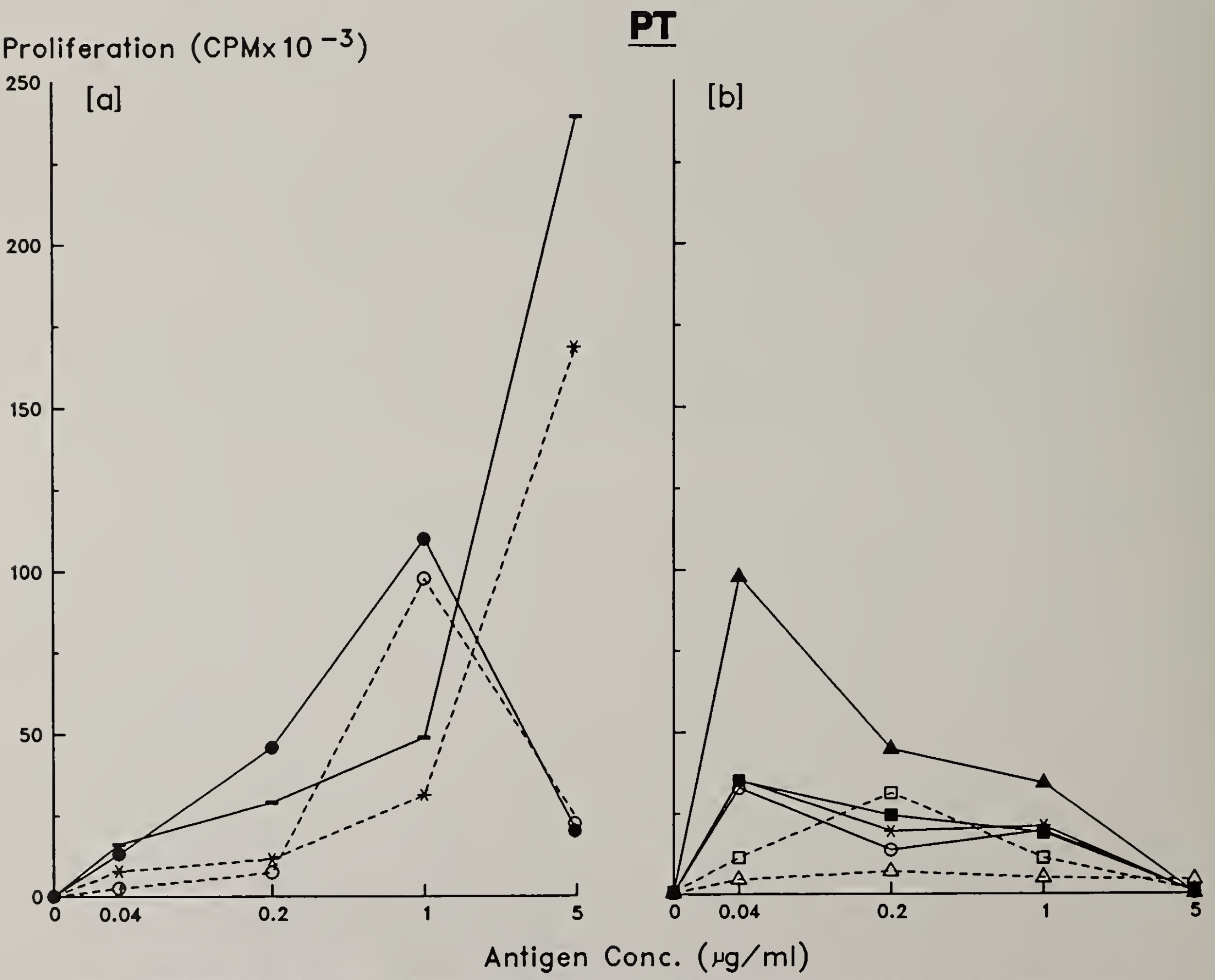

FIG. 3. Proliferative response of spleen cells from aerosol-infected immune and control nonimmune animals to active PT. Six immune (—) and four control (----) animals were tested in three separate experiments where the assay was allowed to proceed for (A) 2 days (–, *) or 3 days (●, ○) or (B) 4 days.

TABLE 1. Effect of aldehyde detoxification of native *B. pertussis* antigens on the in vitro response of T cells from immune animals

		Proliferation[a]		Lymphokine response[a]	
Antigen	Concentration (µg/ ml)	Native antigen	Detoxified antigen	Native antigen	Detoxified antigen
PT	0.04	47,215 ± 3,435	305 ± 217 (0.6%)[b]	6,455 ± 7,075	180 ± 37 (2.7%)
	0.2	24,560 ± 12,070	334 ± 122 (1.3%)	22,509 ± 6,258	208 ± 38 (0.9%)
	1.0	23,422 ± 6,306	403 ± 232 (1.7%)	36,783 ± 6,330	195 ± 50 (0.5%)
	5.0	340 ± 207	74 ± 27 (21%)	39,002 ± 3,381	298 ± 176 (0.7%)
FHA	0.2	16,861 ± 9,643	4,329 ± 2,271 (25%)	20,378 ± 5,959	330 ± 154 (1.6%)
	1.0	23,291 ± 143	4,776 ± 2,557 (20%)	24,266 ± 5,788	1,596 ± 1,048 (6.5%)
69kDa-OMP	0.2	7,095 ± 52,435	3,679 ± 2,435 (52%)	2,838 ± 1,862	653 ± 337 (23%)
	1.0	16,176 ± 1,907	10,256 ± 2,985 (63%)	20,119 ± 3,603	9,624 ± 3,895 (48%)
Medium control		341 ± 152		185 ± 136	

[a] Values represent the mean response in CPM ±SD for spleen cell from 4 aerosol-infected mice; lymphokine release is represented as proliferation in CPM of the IL-2/IL-4 dependent CTLL cell line in CPM.

[b] Values in parentheses represent the response of detoxified antigen as percentage of that with the native antigen.

(data not shown). It is possible that the inactivation processes may have modified T-cell epitopes or processing sites on the native PT. This possibility was examined by testing the activity of detoxified FHA and 69-kDa OMP preparations (Table 1). Formalin detoxification of native 69-kDa OMP only reduced its stimulatory capacity for T-cell proliferation or lymphokine release by 40 to 50%. However, detoxification of FHA reduced its activity by a considerable degree (>90%), especially when lymphokine release was used as the index of T-cell activation.

DISCUSSION

The murine respiratory infection model was used to study cell-mediated immunity to *B. pertussis*. In an examination of the immunological protective mechanisms against *B. pertussis*, it was demonstrated that it is possible to transfer immunity to naive immunosuppressed or nude recipients with T cells from previously infected mice (K. H. G. Mills, A. Barnard, J. Watkins, and K. Redhead, in preparation). The present study of the antigen specificity of the T cells generated by respiratory infection revealed that FHA, 69-kDa OMP, Aggs 2+3, and PT were targets for murine Th-cell recognition. However, the nature of the T-cell response to active PT was ambiguous, and the PTd failed to induce a significant in vitro response.

The demonstration that T cells do play a role in the clearance of bacteria from the lungs of infected mice, as well as the lack of correlation between serological responses and protection in humans (1, 13), underline the importance of identifying immunologically active *B. pertussis* antigens recognized by T cells as well as antibodies for inclusion in acellular vaccines. Epidemiological studies in humans have shown that natural immunity generated by infection with *B. pertussis* is more effective and long lasting than that generated by vaccination (13). Furthermore, it has been demonstrated that, upon rechallenge, mice previously aerosol infected with *B. pertussis* clear the bacteria from their lungs far more rapidly than mice immunized with the whole-cell vaccine (Redhead et al., unpublished). Therefore, the specificity of T cells generated by respiratory infection may provide a basis for identifying protective antigens as components of an acellular *B. pertussis* vaccine.

Proliferative responses of lymphoid cell populations have been used as an index of cell-mediated immunity to purified *B. pertussis* antigens (4, 8, 24). However, a major obstacle to the study of antigen-specific T-cell responses in mice by using proliferation assays has been the mitogenic activity of PT and LPS for murine T and B cells, respectively. This problem has been overcome in part by using T-cell lymphokine release as an index of T-cell activation. Two Th cell subpopulations, Th_1 and Th_2, upon antigen stimulation release IL-2 and IL-4, respectively (14). It is possible to assay both lymphokines, and therefore the activation of both Th subpopulations simultaneously using the CTLL-2 cell line, which is dependent on IL-2 or IL-4 for its growth. Stimulation of spleen cells from naive, nonimmune mice with a variety of *B. pertussis* antigen preparations revealed that only active PT at high concentrations (1 to 5 μg/ml) induced significant T-cell lymphokine responses. In contrast, when proliferation was used as the assay of T-cell activation, heat-treated whole *B. pertussis* organisms, formalin-treated *B. pertussis* sonicate, Aggs 2 + 3, active PT, LPS, and one of two FHA preparations all induced strong responses with normal nonimmune lymphocytes even at low concentrations. Only PTd, recombinant 69-kDa OMP, and the highly purified native 69-kDa OMP and FHA preparations from SmithKline appeared to be free of mitogenic activity.

Using IL-2/IL-4 release as an assay of T-cell activation, it has been shown that FHA was a potent in vitro stimulator of antigen-specific T cells from respiratory-infected mice. Furthermore, the 69-kDa OMP and Aggs 2+3 were recognized by T cells from immune animals. These results also showed that recombinant 69-kDa OMP was capable of inducing comparable responses to the native material. Surprisingly, PTd, considered by many to be the key component of an acellular vaccine, failed to induce a detectable in vitro T-cell response. However, the significantly greater responses of the immune, compared with controls animals to the active PT in the range of 0.04 to 1 μg/ml suggested that PT was recognized in an antigen-specific fashion by T cells from aerosol-infected mice. The lack of nonspecific toxicity of the PTd preparation, taken together with the observation that aldehyde treatment of another purified native *B. pertussis* antigen, FHA, also affected its stimulatory capacity, suggests that the detoxification process may alter PTd's in vitro antigenicity. Previous studies with human peripheral blood lymphocytes from individuals who had suffered from whooping cough also demonstrated weak or undetectable responses to PTd (6, 24). Glutaraldehyde treatment of PT has been shown to modify the antibody response. Neutralizing monoclonal antibodies to native PT recognized conformational determinants, whereas antibodies to PTd recognized denatured proteins and lacked enzyme-neutralizing activity (10). It is possible that inactivation of PT by aldehyde or heat treatment modifies antigen processing sites of epitopes recognized by T cells. Since the mitogenic activity is also affected by these treatments, a nonspecific mechanism such as inhibition of binding to or uptake by the antigen-presenting cells might also be possible.

Although a proportion of the T-cell antigen-specific response may be directed against PT, it may not be a major component of the overall response to *B. pertussis* following respiratory infection. This study failed to demonstrate PT-specific activity in a panel of T-cell lines and clones derived from aerosol-infected mice (K. H. G. Mills et al., unpublished). Furthermore, in a study of the antigen specificity of T-cell clones derived from previously infected humans by re-stimulation of peripheral blood mononuclear cells with formalin-inactivated *B. pertussis,* De Magistris et al. (3) demonstrated that certain clones recognized FHA or the 69-kDa OMP, whereas none were directed against PT. However, PT-specific clones were generated by direct re-stimulation of peripheral blood mononuclear cells with heat-treated PT (2). Although this study could only demonstrate weak antigen-specific responses in mice immunized with PTd (Mills et al., unpublished), other studies (4, 8) have shown that PTd or the whole-cell vaccine can induce T cells that recognize trinitrobenzenesulfonic acid or aldehyde PTd. Furthermore, peripheral blood mononuclear cells from vaccinated individuals have been shown to recognize peptides synthesized according to sequences of the S1 subunit of PT (16). Although PTd has been reported to protect mice against intracerebral challenge by an antibody-mediated mechanism (15), glutaraldehyde, formalin, or heat treatment can reduce the protective activity of PT (5): the role of T cells generated with PTd in protection remains to be defined. Therefore, the use of chemically modified PT in acellular vaccines needs to be carefully considered in terms of its ability to induce optimal immune responses for protection against respiratory challenge.

In conclusion, this study suggests that at least three of the major components considered for inclusion in an acellular *B. pertussis* vaccine (FHA, 69-kDa OMP, and Aggs 2+3) were recognized by antigen-specific memory T cells from respiratory-infected mice. Furthermore, recombinant (69-kDa OMP) or highly purified native

(FHA and 69-kDa OMP) antigens induced consistent T-cell responses in vitro, which were not attributable to mitogenic activity and should not require detoxification for inclusion in an acellular vaccine. In contrast, the antigenicity and immunogenicity of purified PTd for murine T cells was weak or undetectable. The use of PT as a vaccine component may require alternative inactivation methods or the employment of genetically modified recombinant proteins which are devoid of reactogenicity but which retain their immunogenicity.

ACKNOWLEDGMENTS

The authors thank Carine Capiau and Jean Petre at SmithKline RIT, for providing purified *B. pertussis* antigens; Gordon Dougan, Ian Charles, and Neil Fairweather at Wellcome Research Laboratories, Beckenham, United Kingdom, for providing a recombinant 69-kDa OMP; and Andrew Robinson at CAMR Porton Wiltshire for providing Aggs 2 and 3. We also thank Debbie Kirk for typing the manuscript.

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DISCUSSION

DR. SHAHIN: If you add back macrophages to your purified T cells, when you transfer them for protection, can you enhance the clearance of the bacteria?

DR. MILLS: We have not done that but this is a possibility. I am remembering in the first series of experiments, we were looking at depletion of T cells and in the new system we are looking at the addition of purified T cells but I am sure that macrophages do have a role. I think it is not a very simple mechanism of T cell or antibody. It is more complicated mechanisms involving lots of effector cells.

DR. DE MAGISTRIS: I am surprised by your results with the detoxified PT lowering the cell mediated immunity. I wonder whether you used different strains of mice so maybe the T-cell epitopes which were destroyed for one strain of mice, maybe there were other T-cell epitopes which worked in other strains of mice.

DR. MILLS: We were surprised that the PT does completely knock it out but we only looked at one strain of mice, the BALB/c. I would be very surprised if other strains of animals would be found responsive but we have not done that. We may look at other strains.

Cell-Mediated Immune Responses in Patients with Pertussis and in Asymptomatic Exposed Contacts

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The interaction between *Bordetella pertussis* and the immune system is not completely understood. Although humoral responses to several pertussis antigens have been studied following pertussis and pertussis immunization, there is no generally accepted laboratory measure of immunity.

The role of pertussis-specific cell-mediated immunity is unknown in humans. In murine models, cell-mediated immune responses (as measured by cellular proliferation or interleukin-2 production) have been demonstrated to lymphocytosis-promoting factor (LPF) and filamentous hemagglutinin (FHA) following pertussis immunization (2, 3). Specific human T-cell clones from an immune donor proliferated in response to LPF, FHA, and the 69K protein (1). In addition, lymphocytes from convalescent pertussis patients had increased proliferation and/or in vitro immunoglobulin synthesis after stimulation with LPF, A subunit of LPF, B subunit of LPF, and FHA (5).

To further delineate the role of cell-mediated immunity in pertussis infection, the cell-mediated immune responses were studied to a variety of pertussis antigens in subjects with pertussis and their asymptomatic exposed contacts.

MATERIALS AND METHODS

The authors studied 16 subjects with pertussis and 12 of their asymptomatic exposed contacts. The pertussis patients all had cough and were diagnosed on the basis of positive nasopharyngeal cultures, direct fluorescent antibody testing, or serology. A modified enzyme-linked immunosorbent assay measured LPF and FHA immunoglobulin G (IgG) and IgA antibodies and microagglutination was used to determine pertussis agglutinins (4). Positive serologic values were defined as agglutinin titer ≥ 512, or two or more enzyme-linked immunosorbent assay values ≥ 3 standard deviations above age-specific laboratory control values. The pertussis patients ranged from 4 months to 43 years of age (median of 12 years) and had been ill for 2 to 34 days (median of 12 days) when first evaluated. The asymptomatic exposed contacts ranged in age from 2 to 38 years (median of 24 years), had negative nasopharyngeal cultures and direct fluorescent antibody testing, and negative pertussis serologies.

Cellular proliferation was studied with a whole-blood assay. Heparinized whole blood (heparin 10 U/ml) was adjusted to a concentration of 2 x 10^5 mononuclear cells/ml after dilution in RPMI 1640 (Flow Laboratories, Inc, McLean, Va.) with L-glutamine, penicillin, streptomycin, and amphotericin B. Cell suspension (200 µl) was incubated with 20 µl of antigen. Controls were RPMI and phytohemagglutinin M (PHA) diluted 1:4 (Difco Laboratories, Detroit, Mich). Pertussis antigens included LPF, FHA, pertussis endotoxin, B subunit of LPF, and A subunit of LPF (all obtained from List Biologicals, Campbell, Calif.); 69K protein (kindly supplied by Jane V. Scott, Lederle Laboratories, Pearl River, N.Y.); and adenylate cyclase toxin (kindly provided by Erik L. Hewlett, University of Virginia). After a 6-day incubation at 37°C in 5% CO_2, 50 µl of [^{3}H]-thymidine (40 µCi/ml; Amersham International, Amersham, UK) was added to each well; 18 h later the cells were collected on glass fiber filter paper with a Brandel M-24 cell harvester (Gaithersburg, Md.) after washes with 3% acetic acid, distilled water, and 0.5% hydrogen peroxide. The [^{3}H]-thymidine incorporation was determined by measuring the radioactivity of the filter paper disks emitted over 1 min in a Beckman L5800 liquid scintillation

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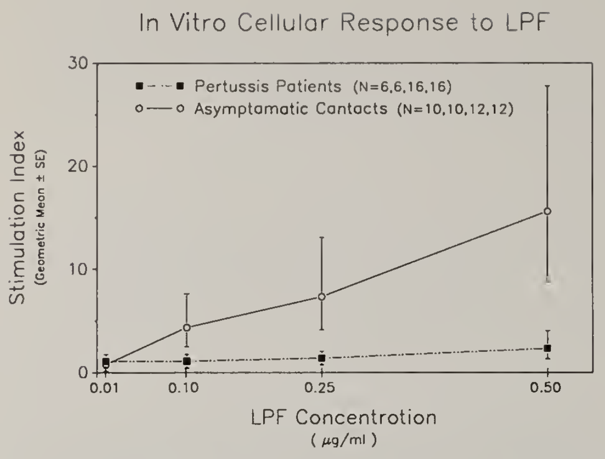

FIG. 1. Stimulation index in response to LPF in pertussis patients and asymptomatic contacts. Asymptomatic contacts had significantly higher mean stimulation indices than pertussis patients at LPF concentrations of 0.10, 0.25, and 0.50 μg/ml ($p < 0.05$).

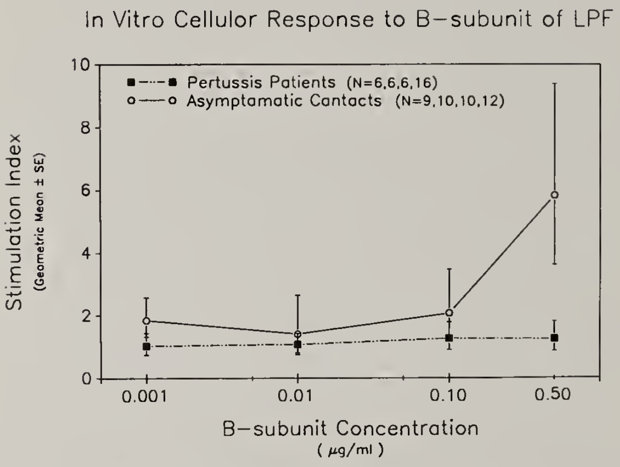

FIG. 2. Stimulation index in response to the B subunit of LPF in pertussis patients and asymptomatic contacts. At a concentration of 0.50 μg/ml, asymptomatic contacts had a significantly higher mean stimulation index compared to pertussis patients ($p = 0.02$).

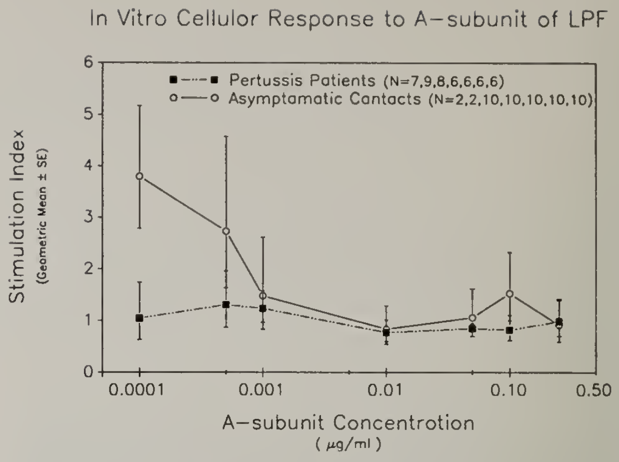

FIG. 3. Stimulation index in response to the A subunit of LPF in pertussis patients and asymptomatic contacts. No significant differences were observed between the two groups.

beta counter (Beckman Instruments, Inc., Palo Alto, Calif.).

The median of triplicate counts was divided by the median of triplicate negative control counts to obtain a stimulation index. Subjects were excluded if the median negative control count was > 1000 cpm. Pertussis patients and asymptomatic contacts were compared by Student *t* test. Pearson correlation measured the association between stimulation index and antibody values, age, and duration of symptoms (all variables were log-transformed prior to analysis).

All blood samples were processed within 24 h of collection. Not all experiments were performed on all subjects due to insufficient blood samples.

RESULTS

Stimulation indices in response to various pertussis antigens are shown in Fig. 1 to 5. At LPF concentrations of 0.10, 0.25, and 0.50 μg/ml, asymptomatic contacts had significantly higher stimulation indices than pertussis patients ($P < 0.05$ Fig. 1). Asymptomatic contacts also had significantly higher stimulation indices than pertussis patients in response to the B subunit of LPF at 0.50 μg/ml (Fig. 2), FHA at 0.50 μg/ml (Fig. 4), and pertussis endotoxin at 0.01 μg/ml (Fig. 5); for all of these $P < 0.05$.

No significant differences between the two groups of patients were noted in response to the A subunit of LPF (Fig. 3). In addition, no significant differences in stimulation indices were noted in response to the 69K protein or adenylate cyclase toxin at concentrations of 0.05, 0.25, and 0.50 μg/ml; however, the number of subjects studied in these experiments was small (2 to 10 subjects per experiment).

Positive control (PHA) responses were similar in both groups. The geometric mean stimulation index (95% confidence interval) was 115.1 (30.0, 442.4) for pertussis patients ($n = 16$) and 104.3 (38.7, 280.9) for the asymptomatic contacts ($n = 12$; $p = 0.90$).

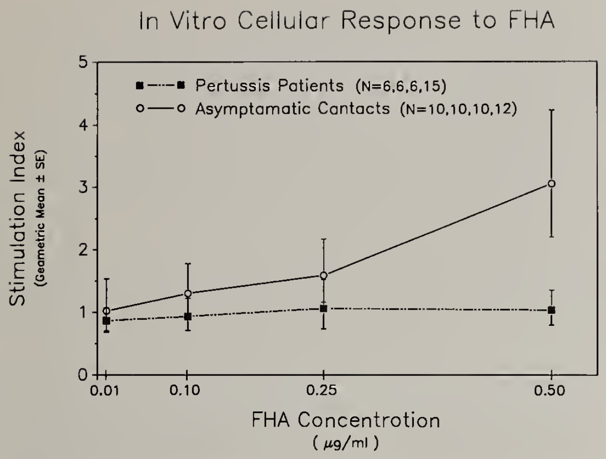

FIG. 4. Stimulation index in response to FHA in pertussis patients and asymptomatic contacts. The mean stimulation index was significantly higher in the asymptomatic contacts compared to the pertussis patients at an FHA concentration of 0.50 μg/ml ($p = 0.02$).

Among pertussis patients, no correlation was noted between stimulation indices and age. Lesser duration of cough correlated with higher stimulation indices in response to LPF at concentrations of 0.50 μg/ml ($r = -0.57$, $p = 0.02$) and 0.25 μg/ml ($r = -0.59$, $p = 0.02$). Higher stimulation indices in response to LPF tended to be associated with lower LPF IgG and IgA antibody values (Table 1). No correlations were noted between stimulation indices in response to the B subunit of LPF and IgG or IgA LPF antibody values or between stimulation indices in response to FHA and IgG or IgA FHA antibody values.

TABLE 1. Correlation between LPF stimulation indices and IgG and IgA antibodies to LPF

LPF concentration (μg/ml)	Antibody type	Pearson correlation	
		r	*P*
0.50	IgG	- 0.45	0.02
0.50	IgA	- 0.41	0.03
0.25	IgG	- 0.43	0.02
0.25	IgA	- 0.44	0.02

Pearson correlation of stimulation index in response to LPF at concentrations noted and LPF antibody values as measured by enzyme-linked immunosorbent assay.

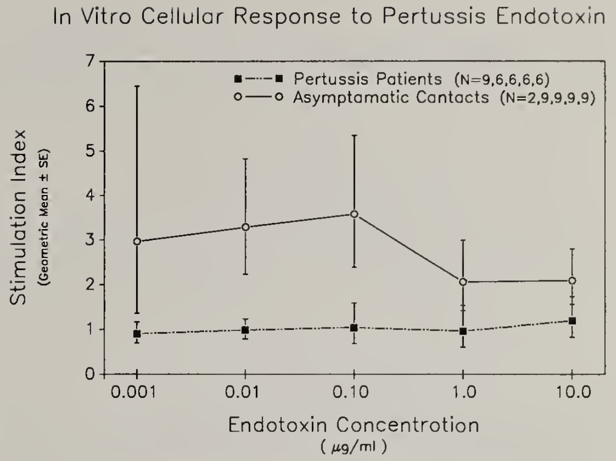

FIG. 5. Stimulation index in response to pertussis endotoxin in pertussis patients and asymptomatic contacts. Asymptomatic contacts had a significantly higher mean stimulation index compared to pertussis patients at an endotoxin concentration of 0.01 μg/ml ($p = 0.02$).

DISCUSSION

Lymphocytes from patients with pertussis and asymptomatic exposed contacts had similar brisk cellular proliferative responses to PHA. In contrast to the PHA responses, asymptomatic contacts had significantly greater cellular proliferative responses to a variety of pertussis antigens than patients with pertussis. There are at least two possible explanations for these differences.

One possible explanation is that the demonstrated cell-mediated immune responses in the asymptomatic contacts were important immunity factors: they were protected because of their lymphocyte reactivity. In contrast, the pertussis patients lacked this reactivity and, at the time of evaluation, had not yet developed it. Serial follow-up studies are needed in pertussis patients to determine the development of specific cell-mediated immune responses.

A second explanation is that the results are due to the assay employed and are not reflective of the true in vivo situation. The whole-blood assay includes subjects' sera as well as mononuclear cells. The B subunit of LPF is a nonspecific mitogen alone and as part of LPF. It is possible that the higher LPF antibody concentrations in pertussis patients' sera neutralized the B subunit and LPF mitogenicity; indeed, there was a trend for subjects with higher LPF antibody values to have lower stimulation indices in response to LPF. However, this hypothesis would not explain the results in the experiments involving FHA which is not known to be a nonspecific mitogen.

Future studies should compare the proliferative responses in the present whole-blood assay with responses utilizing an assay in which the subjects' sera has been removed.

ACKNOWLEDGMENTS

This work was supported by a Gram-Negative Bacterial Infection Award from the National Foundation for Infectious Diseases and by an unrestricted grant from Lederle Laboratories.

We thank Denise Tritt for manuscript preparation.

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DISCUSSION

DR. HUANG: How many of those patients who had the disease still had a lymphocytosis when you did the test?

DR. BLUMBERG: About half had lymphocytosis.

DR. HUANG: Did you have a chance to repeat it after the cell counts were back to normal?

DR. BLUMBERG: We did repeat on some of the patients about a month after the acute evaluation. At that time, not all of the counts were back to normal. One of the preliminary things that we did in this study that I did not show was looking at a higher concentration in the assay of cells in pertussis patients since a lot of them did have lymphocytosis. We used $2x10^6$ cells in some of the assays and when we compared this to two $2x10^5$ cells we got very inconsistent results. I am not sure why but in the more concentrated blood maybe there was too many red cells in the wells that interfered with the antigen.

Immunoglobulin E Response to *Bordetella pertussis* Filamentous Hemagglutinin and Pertussis Toxin in Mice

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The reaginic adjuvant effect of pertussis vaccine (PV) has been well documented (4, 6, 18), and several studies have suggested the importance of pertussis toxin (PT) as the component responsible for this adjuvanticity (11, 15, 20).

Before the present study, there were only two reports of an immunoglobulin E (IgE) response to *Bordetella pertussis*. Pauwels et al. (24) immunized Wistar rats with a mixture of PV and $Al(OH)_3$ and, using a solid-phase radioimmunoassay procedure and whole *B. pertussis* cells as the antigen, observed an IgE response to undefined components of the bacterium. Later, Finger and Wirsing von König (5) employed an enzyme-linked immunosorbent assay with whole cells of *B. pertussis* to investigate IgE responses in whooping cough convalescent sera, but only 3% of positives were detected. Neither of the above studies identified the specific IgE-inducing antigens of *B. pertussis*.

The object of the present investigation was to determine whether IgE antibodies could be produced in mice against two of the *B. pertussis* antigens, PT and filamentous hemagglutinin (FHA), which are major components of acellular vaccines.

MATERIALS AND METHODS

Animals. Adult Ham/ICR mice (Charles River U.K. Ltd., Margate, England) bred in the authors' facility, were used at 4 to 6 weeks of age for immunization and at 6 to 8 weeks for passive cutaneous anaphylaxis (PCA).

Bacteria. *B. pertussis* BP353 (FHA^-) and BP357 (PT^-), as described by Weiss et al. (30), were obtained from the departmental culture collection. They were maintained on Bordet-Gengou agar plates containing 50 µg/ml kanamycin (Sigma Chemical Co., St. Louis, Mo.).

***B. pertussis* antigens.** *B. pertussis* BP353 was grown in liquid cyclodextrin medium (10) at 37°C for 48 h with shaking, and PT was extracted from the supernatant by a dye-ligand affinity chromatography method modified from Sekura et al. (26). Briefly, the supernatant was mixed overnight at 4°C with Blue Sepharose CL-6B (Pharmacia LKB) in the presence of 1 mM phenyl methyl sulphonyl fluoride (Sigma) as a protease inhibitor. The gel was then filtered through a G1 sintered glass funnel (Gallenkamp), packed into an LKB 2137 chromatography column (2.6 x 35 cm, Pharmacia LKB), and washed with 0.05 M Tris-HCl buffer pH 8.0. PT was eluted with 0.05 M Tris-HCl pH 8.0 containing 1.0 M NaCl, and 10-ml fractions collected with an Ultrorac fraction collector connected to a Uvicord II-UV absorptiometer (Pharmacia LKB) to monitor the elution peak. The same method was used for extracting FHA from *B. pertussis* BP357 culture supernatants. The protein content of the samples was measured by the method of Lowry et al. (17), and purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1). The PT preparations showed the five characteristic bands of molecular weight 13 to 29 kDa together with an estimated 10% of impurities. In contrast, the FHA preparations showed one large band at about 200 kDa and very little else. The PT and FHA were dialyzed against physiological saline before injection into mice. A carbodiimide toxoid (PTd) of PT in 1:1 ratio with FHA was made by the method of Christodoulides et al. (3).

Immunization. To find suitable conditions for raising anti-FHA IgE, three tenfold spaced doses of FHA were administered to mice in a protocol involving three injections over 56 days. This dosage was modified from Levine and Vaz (16) who used $Al(OH)_3$ as the IgE adjuvant. Groups of 20 Ham/ICR mice, 4 to 6 weeks old, were injected intraperitoneally on day 0 with 0.1, 1.0, or 10.0 µg FHA with 1 µg PT. This injection was followed by intraperitoneal booster doses of the same amounts of FHA alone on days 28 and 56 and bleeding on day 63. To raise IgE-containing anti-PT sera, the same protocol was used except that the immunizing antigen was a 1:1 ratio FHA:PTd, again used in doses of 0.1, 1.0, and 10.0

μg of total protein with 1 μg of bioactive PT as adjuvant for the first injection. Anti ovalbumin (OA) IgE was used as a positive control serum for the PCA test; it was administered by the same three-injection scheme with 0.1 μg of OA (Sigma, 5x crystallized) and 1 μg of PT, and the pooled blood of 20 mice were stored as serum. All the other mouse sera were collected and stored individually. The PCA tests, however, were usually done on pools of 10.

PCA. PCA tests were done in 6- to 8-week-old Ham/ICR mice of either sex and body weight more than 40 g by a method based on that of Ovary (23) and reviewed by Watanabe and Ovary (29). One significant deviation from this method was the use of four intradermal injection sites per mouse so as to have a positive control site (anti-OA serum) and three experimental sites for *B. pertussis* sera. Ten mice were used in a typical set of PCA tests. Hair was clipped from the dorsal surface of each mouse and 50-μl doses of the test sera were injected intradermally into each animal under Hypnorm (Janssen Animal Health) analgesia. The injection site of the positive serum was varied systematically among mice. Each experimental serum was usually tested in quadruplicate, with each replication given in a different animal.

To elicit the PCA reactions, the sensitized mice were challenged intravenously at intervals ranging from 2 to 7 days with a mixture of either FHA or PT mixed with OA and Evans Blue (Sigma). The challenge mixture was made in phosphate-buffered saline so that each 0.2 ml contained 0.5 mg FHA or PT plus 1 mg each of OA and the dye. At 30 min after challenge, the animals were killed and the skins removed so that the diameter of blueing, where the dye had leaked, could be measured on the inner surface. The diameter of each blue zone was measured in two directions at right angles and the average taken. The averages of zones from different mice were summarized as arithmetic means with 95% confidence limits. On the rare occasions when the anti-OA site gave an unexplained negative result, all the observations from that animal were discarded.

RESULTS

IgE adjuvanticity of PT towards OA. Before attempting to raise IgE-containing serum to FHA and PT, extensive prior studies were made in Ham/ICR mice using OA as a model antigen and PT as the IgE adjuvant. Strongly IgE-positive anti-OA sera were obtained using the three-injection protocol described above. These sera were used as a positive control at a 1:20 dilution in subsequent PCA tests with FHA and PT and gave a convincingly positive (average of 10 mm) PCA reaction even at a 1:70 dilution. The control serum which had been produced with 0.1 μg of OA at each of the three immunizing doses was slightly more active than a pooled serum made with three 1.0-μg doses of OA. When the immunizing doses of OA were increased to 10 μg each, the pooled serum was PCA-negative.

Effect of challenge dose and sensitization time in the PCA with OA. Using a constant 1:20 dilution of the IgE-containing anti-OA serum and a 2-day sensitization interval, mice were challenged with Evans Blue mixed with doses of OA varying between 62.5 μg, and 2.5 mg in twofold steps. Strongly positive PCA results were obtained with challenge doses of 2.5, 1.0, and 0.5 mg; at 250 μg, the reaction was weaker, and at 125 and 62.5 μg, the reactions were too faint to be usable. Subsequently, 1.0 mg was used as the standard challenge dose of OA.

As described below, problems of generalized blueing of the whole skin of the mouse were encountered in the PCA tests with FHA and PT as the challenge antigens. To overcome this problem, experiments were done in parallel in the OA system to find out whether the normal 2-day interval between skin sensitization and challenge could be extended to 7 days without loss of PCA activity.

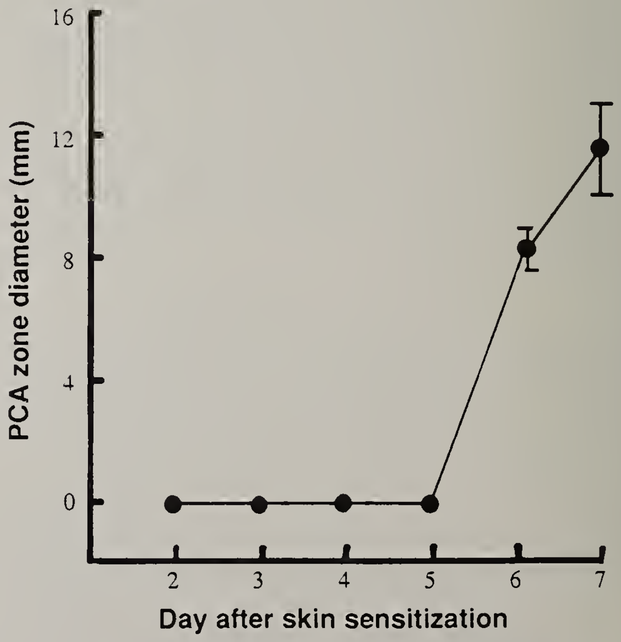

FIG. 1. The beneficial effect of a 7-day interval between skin sensitization and challenge in PCA tests with FHA in mice. Error bars indicate 95% confidence limits.

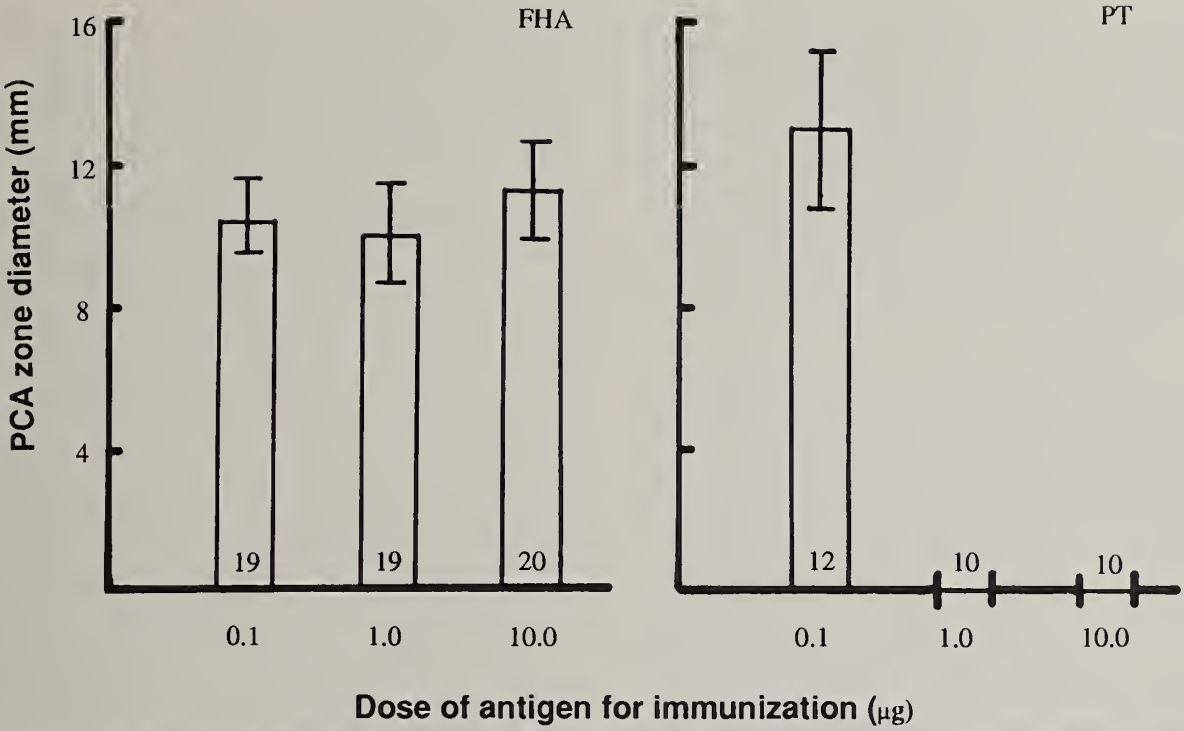

Fig. 2. Application of the 7-day PCA test to detect IgE antibodies to FHA and PT in mice. Sera were raised by a three-dose protocol over 63 days, with the immunizing doses indicated on the abscissa. Figures inside the bars indicate the numbers of PCA zones observed, and error bars indicate 95% confidence limits.

The average zones with the 7-day interval were no smaller in size and tended to be more sharply defined.

Beneficial effect of a 7-day interval in the PCA test with FHA. In the early attempts to obtain a PCA response with pooled anti-FHA sera and FHA, the usual 2-day sensitization interval was used, but with very variable results. Most mice showed a diffuse blueing over the whole skin, with only the occasional animal exhibiting a slightly darker region at the site of serum injection. This result was interpreted as due to an interfering effect of antibodies other than IgE. Experiments were therefore done to examine the effect of increasing the interval between sensitization and challenge from 2 to 7 days. Fig. 1 shows that this increase had a highly beneficial effect on the PCA test with FHA: the longer interval gave an average zone diameter of 12 mm, whereas intervals between 2 and 5 days gave no reaction at all. Also, the diffuse blueing had ceased to be a problem.

Additional evidence that these 7-day PCA reactions were due to IgE was obtained by demonstrating their absence (six replicates) when the anti-FHA serum was heated at 56°C for 1 h. A similar loss of PCA reactivity was found with the anti-OA serum (15 replicates). All of these negative results were observed in mice whose skin elsewhere had positive OA-anti-OA and/or FHA-anti-FHA PCA reactions.

Detection of anti-FHA and anti-PT IgE by 7-day PCA. Using the 7-day PCA test, experiments were done to determine the effect of different doses of FHA and FHA-PTd on the production of IgE antibodies to FHA and PT antigens in the three-injection protocol. As shown in Fig. 2, anti-FHA IgE was produced over a wide range of immunizing doses of FHA, from 0.1 to 10 µg, whereas anti-PT IgE was produced only after the lowest (0.1 µg of antigen) of the three doses tested.

DISCUSSION

Although a wide variety of protein antigens can induce IgE antibodies in a range of mammalian species (12, 14, 19), particular conditions of immunization may be needed for their demonstration. Factors that favor IgE production include the use of minute but repeated doses of antigen, the admixture of the first dose of antigen with $Al(OH)_3$, the use of PV or PT as adjuvants, and the choice of genetically appropriate recipient animals. Ham/ICR mice were previously shown (28) to be IgE producers to OA when PV was employed as adjuvant. In the present study, we found that PV could be replaced by PT to induce anti-OA IgE. In the three-injection scheme of Levine and Vaz (16), the dose of PT is not crucial within the range 0.001 to 1 µg, for addition to OA. Thus, the benefit of minute (0.1 µg) OA over larger doses at each of the three injections was confirmed (2, 13, 25, 27). Recently, Ohmori (22) obtained similar results with a hapten-antigen conjugate. On the other hand, Lehrer et al. (15), in a one-dose immunization procedure with ovalbumin and a crude extract of PT as adjuvant, found high titers of both IgE and IgG; the latter interfered with the 2-day PCA test.

With the *B. pertussis* antigens as inducers of IgE, PTd behaved similarly to OA in stimulating IgE when used at 0.1 µg in each of the three doses (with 1.0 µg of bioreactive PT in the first dose); FHA did not. In the FHA system, the three-injection scheme at all dose levels (0.1, 1.0, and 10.0 µg) gave qualitatively similar responses, and it would be necessary to extend the dose range in both directions to define the limits for anti-FHA IgE production in these mice.

PCA was chosen as the IgE-detection method only after failing to obtain satisfactory results by enzyme-linked immunosorbent assay: the fault seemed to lie in the inadequacy of commercially available anti-mouse and anti-rat IgE sera, which gave rise to problems of high background. With the normal 2-day PCA test, there were no difficulties in demonstrating the presence of IgE to OA in Ham/ICR mice. However, with anti-FHA sera for skin sensitization and an FHA intravenous challenge, the mice developed a generalized blueing over the whole skin which obscured the PCA reaction. In a sense, this was a similar high-background problem to that encountered with enzyme-linked immunosorbent assay. Clausen et al. (4) noted a generalized blueing in mice that had been sensitized with serum containing high concentrations of immunoglobulin G1 (IgG1). This blueing was overcome by extending the 2-day interval between skin sensitization and intravenous challenge to 7 days. Ovary (23) had previously shown that IgE could still be detected in the skin at 14 days; Hirano et al. (9) reported that the half-life of intradermally injected IgE antibody was 6 days. We found a beneficial reduction in the background blueing by extending the interval of the PCA test to 7 days.

For induction of IgE to PT, FHA/PTd (0.1, 1.0, or 10.0 µg) as a source of nontoxic PT antigen was mixed with bioactive PT (1.0 µg) used as adjuvant. Doses of PT above about 2.5 µg were lethal. For challenge in the PCA, the massively larger dose of 1.0 mg could be injected intravenously since the test lasted only 30 min. PTd seemed to differ from FHA in not producing IgE in the three-injection schedule at doses above 0.1 µg.

After this investigation had started, there were reports of IgE responses to *B. pertussis* in humans. Using the RAST assay, Haus et al. (7) showed that there was an IgE response to *B. pertussis* whole cells in infants after DTP vaccination. They speculated that, because of the high risk of allergy in certain individuals, the administration of the vaccine should be delayed. In 1989, Wirsing von König and Finger (31), with an enzyme-linked immunosorbent assay and whole cells of *B. pertussis* as antigen, detected IgE antibodies in only a small percentage of individuals. They concluded that IgE did not have any effect on disease outcome or course. Most recently, Hedenskog et al. (8) detected with RAST an anti-PT IgE response during pertussis infection and after immunization with both an acellular and a whole-cell vaccine. During pertussis infection and after immunization, 65% and 35%, respectively, of the individuals tested exhibited an anti-PT IgE response. In mice, Munoz and Peacock (21) did not detect production of anti-PT IgE under conditions in which anti-OA IgE was detected.

It seems, therefore, that there may be a significant incidence of human IgE responders to PT, and it will be of interest to find out whether other *B. pertussis* antigens such as FHA are also IgE inducers. The immunological significance of such observations may be more difficult to evaluate.

ACKNOWLEDGMENTS

This work was done during the tenure by D.S.J.L. on a studentship from the Medical Research Council; this assistance is gratefully acknowledged.

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DISCUSSION

DR. SHAHIN: I was not clear about one of your later slides. Did you see IgE to FHA when you injected FHA alone or was the --

DR. LINDSAY: It was FHA plus PT.

DR. SHAHIN: In the old literature there is a suggestion that if you give multiple, very small doses of antigen you can also elicit IgE responses. Did you ever see anything with FHA by itself?

DR. LINDSAY: I did not, it had to be with the adjuvant or the PT.

Immunoglobulin E Response to Pertussis Toxin after Vaccination with Acellular Pertussis Vaccine

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The ability of pertussis toxin (PT) to act as an adjuvant enhancing the immunoglobulin E (IgE) synthesis is known from experimental studies (6), but the effects on humans are less well known. One report suggests that pertussis vaccine may induce production of IgE antibodies to whole *Bordetella pertussis* bacteria (4). IgE responses to specific antigens of *B. pertussis* have been little studied.

In a pilot study, the IgE antibody responses to PT in whooping cough and after pertussis vaccination were investigated (5). The vaccine response was studied in 15 of 304 children who had participated in a clinical trial primarily immunized with either a whole-cell or two-component acellular vaccine and given a booster dose of the acellular vaccine (1, 2). The results of the pilot study indicated a greater IgE antibody response after the acellular booster dose in the children previously immunized with acellular vaccine as compared with the former whole-cell vaccinees. Furthermore, the results from the clinical trial showed that local reactions were significantly more common after the booster dose in the former acellular than in the former whole-cell vaccine recipients.

The findings raised the question of whether the IgE response to PT correlated with the appearance of local reactions. The aim of the present study was to analyze the possibility of such a relationship.

MATERIALS AND METHODS

Participants. As shown in Table 1, in a phase-II clinical trial, 304 infants aged 6 to 8 months received either two or three doses of an adsorbed two-component acellular pertussis vaccine or three doses of a plain whole-cell vaccine (2). At the age of 2 years (range: 23 to 26 months), 241 of 304 (79%) children were given a booster dose of the acellular vaccine (1). Of the 241 children, 99 (41%) were selected according to the following inclusion criteria: (i) primary immunization with two or three doses of the acellular pertussis vaccine and no local or systemic reactions following the acellular booster dose ($n = 40$), (ii) primary immunization with two or three doses of the acellular pertussis vaccine and both redness and swelling within 14 days after the booster dose ($n = 30$), and (iii) primary immunization with three doses of the plain whole-cell pertussis vaccine and a booster dose of the acellular vaccine, regardless of adverse reactions ($n = 29$).

TABLE 1. Study population

Type of primary immunization at 6 to 8 months of age	AA/A[a]	WWW[b]
No. of infants with primary immunization	231	73
Type of booster immunization at 24 months of age	A[c]	A
No. of children:		
Given a booster dose	212	29
With redness and swelling	30 (14%)	1 (3%)
Without any reaction	40 (19%)	6 (21%)
Excluded	142 (67%)	0

[a] Two or three doses of acellular vaccine.
[b] Three doses of whole-cell vaccine.
[c] One dose of acellular vaccine.

Vaccines. Two types of vaccine were given for primary immunization as described previously (2); i.e., a two-component aluminum-adsorbed acellular vaccine (JNIH-6, Japan National Institute of Health, Japan) containing equal amounts of detoxified PT and filamentous

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* Presenting author.

TABLE 2. IgE response to PT after a booster dose of acellular pertussis vaccine in relation to local reactions in 2-year-olds previously immunized at 6 to 8 months with two or three doses of acellular vaccine

Blood sample	Reactions to booster	No. of positive[a] samples/total no. of samples (%)	*P* value	Median antibody level in positive samples (PRU/ml)	*P* value
Before booster	(+)[b]	13/30 (43)	0.03	0.34	> 0.05
	(–)[c]	8/40 (20)		0.34	
After booster	(+)	29/29 (100)	0.006	1.90	0.007
	(–)	29/40 (73)		0.60	

[a] ≥ 0.1 PRU/ml IgE to pertussis toxin.
[b] (+) Local reaction with redness and swelling within 14 days.
[c] (–) No local or systemic reaction.

hemagglutinin and a nonadsorbed whole-cell vaccine (Wellcome Pertussis Vaccine BP Per/Vac, Wellcome Foundation LTD, London). For the booster dose, the same lot of the acellular vaccine JNIH-6 was administered.

Vaccination. For the booster vaccination, a single dose of 0.5 ml of JNIH-6 was administered deep-subcutaneously at the lateral aspect of the thigh. A dose was postponed if the child had an acute infection with a temperature >37.9°C or received antibiotic therapy on the day of immunization. No other vaccinations were given within 2 weeks.

Registration of adverse reactions. The method for registration of adverse reactions has been described earlier (1). Briefly, the parents were given standardized questions by telephone the day after the vaccination and by personal contact 14 days after vaccination at the time of the blood sampling.

Blood sampling. A capillary blood sample of at least 0.4 ml of whole blood was drawn from the fingertip in microtainer tubes (Becton Dickinson and Co., Rutherford, N. J.) prior to vaccination, and another sample was obtained 2 weeks after the booster dose. The serum samples were stored at -20°C.

Laboratory methods. IgE antibodies against PT were measured with a modified procedure of the Phadebas RAST^{TM} (Pharmacia, Uppsala, Sweden) assay. The PT preparation (Kanonji Institute, Osaka University, Japan) was coupled to CnBr-activated discs by the Special Allergen Service (Pharmacia). The test was then done according to the manufacturer's instructions, except that the incubation period was extended to 24 h under continuous shaking (3). All sera were diluted 1:2 in phosphate-buffered saline. The reference serum was diluted to 0.05, 0.1, 0.35, and 17.5 Phadebas RAST Units (PRU). Values of 0.1 and above were regarded as positive, corresponding to a cutoff twice that of the detection limit in the assay. The intra- and interassay variations for the 1:2 dilution of samples used were determined in five experiments to be 5.7% and 7.1%, respectively.

Analysis of IgG antibodies by a neutralization test in Chinese hamster ovary cells (2) was performed prior to the IgE antibody assay.

Computer program and statistical methods. Data were processed and analyzed on a microcomputer by means of a software program, Quest, developed at the Department of Preventive Social Medicine at the University of Umeå, Sweden. Chi-square tabulations were calculated when comparing two independent proportions. The Mc Nemars test was used to compare two dependent proportions. The significance of differences between titer levels was evaluated by the Mann-Whitney test. Unmatched distributions of titers were tested by the Kolmogorov two-sample test; matched distributions were tested by the Sign test.

RESULTS

Prebooster samples. A positive (≥ 0.1 PRU/ml) IgE antibody response to PT before the booster dose was slightly more common in the former acellular vaccinees who reacted with both redness and swelling after the booster injection than in the acellular vaccinees who showed no reactions (Table 2). There was no difference in the rate of positive titers between the former whole-cell vaccinees and acellular vaccine recipients (Table 3).

The distribution of IgE antibody levels (Fig. 1) did not differ among varied vaccination regimens ($P > 0.8$) nor

TABLE 3. IgE response to PT before and after a booster dose of acellular pertussis vaccine in relation to primary vaccination protocol

Blood sample	Primary immunization	No. of positive[a] samples/total no. of samples (%)	*P* value	Median antibody level in positive samples (PRU/ml)	*P* value
Before booster	AA/A[b]	21/70 (30)	> 0.05	0.34	> 0.05
	WWW[c]	10/29 (34)		0.24	
After booster	AA/A	58/69 (84)	< 0.001	0.76	0.003
	WWW	8/28 (29)		0.16	

[a] ≥ 0.1 PRU/ml IgE to pertussis toxin.
[b] AA/A, Two or three doses of acellular vaccine.
[c] WWW, Three doses of whole-cell vaccine.

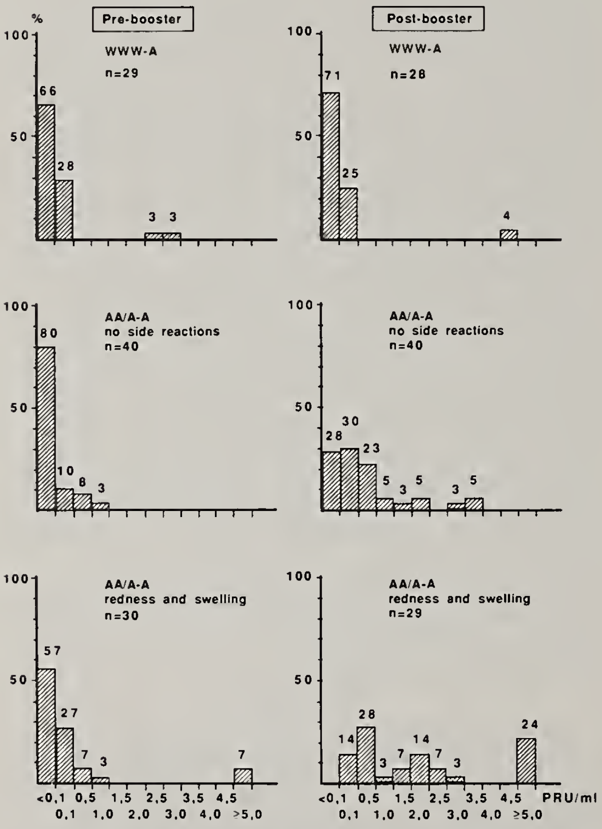

FIG. 1. Distribution of IgE antibodies to PT in blood samples drawn from 2-year-olds before and 2 weeks after a booster dose of an acellular pertussis vaccine in relation to primary vaccination regimen and side reactions to the booster dose. WWW, primary vaccination with three doses of whole-cell vaccine; AA/A, primary vaccination with two or three doses of acellular vaccine; A, one booster dose of acellular vaccine. The concentration of IgE antibodies is given as PRU/ml.

between groups with appearance or absence of local reactions after the booster dose ($P = 0.31$). The median IgE antibody levels in the positive samples (Table 2 and 3) did not differ significantly in the three groups.

Postbooster samples. All children (100%) who were immunized with the acellular vaccine for primary series and reacted with redness and swelling after the booster dose had positive IgE antibody levels to PT. This rate differed significantly from the 73% IgE reactions among the children without any adverse reactions (Table 2).

In the group of children who had received whole-cell vaccine for primary vaccination, the rate of positive IgE antibody levels after the booster dose was significantly lower than in the former acellular vaccinees (Table 3).

The median titer in the positive samples was significantly higher in the group of children with local reactions than in the group without any reactions (Table 2). It was also higher in the entire group of acellular vaccine recipients than in the group of whole-cell vaccinees (Table 3).

Comparison of pre- and postbooster samples. The rate of positive IgE antibodies (Table 2 and 3) rose significantly after the booster dose in the former acellular vaccinees who reacted with redness and swelling and in the group without reactions ($P < 0.001$). The predictive value (defined as the proportion of those testing positive for IgE in the prebooster sample who were true positive, i.e., showing local reactions after the booster) of a positive IgE antibody titer for local reactions was only 62%. In the former whole-cell vaccinees, the rate of positive IgE antibodies was unchanged ($P = 0.69$).

There was a significant change in the distribution of titers (Fig. 1) before and after the booster dose in the acellular vaccine groups ($P < 0.001$), but not in the former whole-cell vaccinees ($P = 0.77$). There was no significant difference in IgE antibody response in the pre- or postbooster samples between children who had received two or three doses of acellular vaccine for the primary series. The IgE response to PT was found to rise independently of IgG antibodies to PT as measured in the Chinese hamster ovary neutralization assay (data not shown).

DISCUSSION

The present study demonstrated that specific IgE antibodies to PT were significantly more common after a booster dose of acellular pertussis vaccine in children who had received the same acellular vaccine for the primary series than in children who had been vaccinated with a whole-cell vaccine previously. Also, the titer levels in children with an IgE response were higher in the former acellular vaccinees than in the prior whole-cell vaccine recipients.

The former acellular vaccinees who had reacted with both redness and swelling after the booster dose had IgE antibodies to PT significantly more often than those who reported no reactions at all. The IgE antibody responses were also significantly higher in those with reactions versus those without.

The most striking difference in IgE response to PT was noted between children who had received whole-cell vaccine and those who had received acellular vaccine for primary vaccination. The former whole-cell vaccinees maintained a low rate of IgE antibodies, whereas the majority of the former acellular vaccinees developed an IgE response.

It should be noted that the former whole-cell vaccinees represented the entire group of vaccinees without any selection for local reactions. On the other hand, only 1 out of the 29 children had a local reaction with both redness and swelling.

The vaccines given for primary vaccination differ both in antigen composition and antigen content. Further, the whole-cell vaccine is a nonadsorbed, plain preparation; the acellular vaccine is adsorbed to an aluminum carrier. In a study of immune responses to tetanus toxoid vaccine, IgE responses were noted to an adsorbed preparation and not to a nonadsorbed tetanus toxoid (7). Also, as in the present study, the IgE responses did not parallel the IgG responses as measured by neutralizing antibodies to tetanus toxin.

The study findings indicate a possible relation between the presence of IgE antibodies to PT and local reactions. It was shown that IgE antibody responses to PT after a booster dose of acellular vaccine were more common and more pronounced in children previously immunized with an acellular vaccine than with a whole-cell vaccine. Since the two types of vaccine also differed in their aluminum content, the relative importance of each factor is difficult to discern.

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DISCUSSION

DR. RELYVELD: I do not agree with the last sentence of your abstract stating the two vaccines also differ in aluminum content. I think that you should clearly mention that the component vaccine contains aluminum and the whole cell vaccine does not contain aluminum as you said before. It is known that aluminum adjuvants give rise in IgE antibodies to various antigens including, toxoids or allergens.

DR. Y. SATO: The same comment. The acellular vaccine contains aluminum and the whole cell vaccine does not. So it is different.

DR. BLENNOW: There is a difference in the antigen content also --

DR. Y. SATO: I mean the aluminum.

DR. BLENNOW: Of course, there is no aluminum content in the acellular vaccine.

DR. MEADE: The vaccine you were using was JNIH-6, is that correct?

DR. BLENNOW: Yes, JNIH-6.

DR. MEADE: What can you tell us about the amount of bioactive toxin in that vaccine at the various times it was being used? How much bioactive pertussis toxin was in that vaccine at the time it was being used in the studies?

DR. BLENNOW: I believe it was 7.5 µg.

DR. MEADE: I am talking about active pertussis toxin.

DR. BLENNOW: I do not know. Dr. Sato may know.

DR. MEADE: Was there active pertussis toxin? At the beginning of the trial those vaccines were carefully studied and there was no active toxin or very little active toxin in the vaccine. At the time when the boost was given, is it possible that there was a greater amount of active toxin due to reversion of the vaccine?

DR. Y. SATO: I am not sure but I checked again and again. How much active toxin is not clear. About 0.1 microgram protein active pertussis toxin.

DR. MEADE: Well, in the specific vaccine used in the trial, this occurred over a very long time from the time of the first immunization until the last immunization. Was there increased amounts of active pertussis toxin later in the study? Was that documented?

DR. BLENNOW: There was a little bit of residual activity in the vaccine but as far as I know, this did not change throughout as the vaccine was kept, it was stored.

DR. SIBER: We do not have human data, but we had performed studies in mice that are relevant to the issue of whether you need aluminum for this effect. In mice when you give 100 nanograms of intravenous pertussis toxin simultaneously with non-adsorbed tetanus toxoid, you induce an IgE and IgG-1 response with primary immunization and then when you come back with a tetanus toxoid alone as much as six months later you still have an enhanced IgE and IgG-1 response in the mice that saw pertussis toxin as the primary immunization. There is no alum in that system at all.

DR. SHAHIN: Dr. Siber, have you looked for interleukin-IV after you give your PT in those mice?

DR. SIBER: We have inhibited that system with anti-IL-IV monoclonal antibodies, and what we see is inhibition of the IgE but not the IgG-1.

DR. BOUX: When you measured the IgE, was that antigen specific and did you look at total IgE?

DR. BLENNOW: We did not measure total IgE. We measured only IgE against pertussis toxin.

DR. BOUX: I think you answered the next question. Have you done any preliminary investigation about IgE to other components such as FHA in that vaccine?

DR. BLENNOW: We did not.

DR. BOUX: And did you have a chance to look at the JNIH-7 sera at all?

DR. BLENNOW: We have not so far but we might be able to.

DR. CRYZ: You said that the elevated levels of IgE prior to boosting had a 62 percent predictive value of having an adverse reaction. Is that correct?

DR. BLENNOW: Yes.

DR. CRYZ: So I am taking that to mean your conclusion is that pre-existing IgG is responsible for the increased adverse reactions. Is that the take-home message?

DR. BLENNOW: Yes.

DR. CRYZ: We have immunized a large number and there was no increased adverse reactions compared to their primary immunization so it is two different antigens. The second thing I would like to ask you is did you look post-boosting at antibody levels induced to pertussis toxin and have you seen a difference in the group that had higher rates of reactions?

DR. BLENNOW: We did. Children primed with the whole cell vaccine, after an acellular booster they had extremely high titers in the CHO cell assay, much, much higher than those who had their fourth acellular vaccine dose and there was no correlation between the antibodies to pertussis toxin measured by the CHO cell and the IgE response.

DR. CRYZ: But independent of the CHO cell assay, did you quantitate how many micrograms of antibody was induced by the boosting?

DR. BLENNOW: No.

DR. HUANG: You said you read the skin test 14 days after the booster. That is fairly long for the IgE response to develop. What is the average onset of the reaction?

DR. BLENNOW: We measured the adverse reactions on two occasions and one was 24 hours after the booster dose and the second one was 14 days after. Most of these reactions appeared during the first 24 hours.

Live Oral Attenuated *Salmonella typhimurium* Vaccine Vectors Which Induce Formation of Antibody to *Bordetella pertussis*

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Pertussis vaccines currently in use and under test are intended for parenteral administration. However, *Bordetella pertussis* is a pathogen of respiratory mucosal surfaces, and secreted or exuded antibodies might be protective. Thus, a vaccine that induces mucosal immunity might provide an alternative or supplement to parenteral vaccines. Live attenuated vaccines administered intranasally for respiratory tract diseases such as influenza are effective (17). However, respiratory administration of *B. pertussis* would require construction of a suitable attenuated mutant strain and might present technical problems involved with administration and storage.

Live oral attenuated *Salmonella* spp. vaccine vectors containing plasmid-borne recombinant genes have been described for several antigens (5). We have begun to test such systems for oral vaccination against pertussis. Gut mucosal antigen sampling occurs constantly via specialized cells (14). As antigen-stimulated lymphoid cells differentiate, they migrate through the body with many eventually returning to the gut as immunoglobulin A (IgA) secreting cells (1). Some cells exposed to antigen in the gut migrate to other mucosal surfaces, such as the respiratory tract and genitourinary tract, and may secrete antigen-specific IgA there (11).

Oral vaccination with attenuated *Salmonella typhimurium aroA*$^-$ strains leads to both antibody and cell-mediated immunity (2). Although cell-mediated immunity is not known to play a role in immunity to pertussis, it may do so. Recent reports of sequestration of *B. pertussis* in mononuclear cells (7) suggests that cell-mediated immunity could potentially speed recovery or prevent long-term carriage.

In the studies reported here, three attenuated *Salmonella* vectors expressing plasmid-encoded, truncated portions of filamentous hemagglutinin (FHA) proteins encoded by *fhaB* (3, 15) were compared for their ability to induce specific antibody formation in a mouse oral immunization model. In preliminary studies using an *aroA* deletion mutant of *Salmonella dublin* (12), it was found that recombinant plasmids were cured rapidly in the mouse intestine. To obtain sufficient FHA antigen expression for antibody induction, it was necessary to select for plasmid maintenance using oral tetracycline (TC) (12). Therefore, two additional *Salmonella typhimurium* vectors were compared with the *S. dublin* vector. These *S. typhimurium* strains were isogenic, except that one contained the same *aroA* deletion as the originally described *aro*-dependent *S. typhimurium* (8), while the other contained attenuating deletions in both *cya* (adenylate cyclase) and *crp* (cAMP receptor protein) (4). Both strains were also deleted for the *asd* gene (13), thus requiring diaminopimelic acid (DAP) for growth. Recombinant plasmids in the *S. typhimurium* strains contained a copy of the *S. typhimurium asd* gene, to allow growth in the absence of DAP. This *asd*$^-$ bacterial vector-*asd*$^+$ plasmid system has two additional advantages, in that (i) cells cured of the plasmid make abnormal cell walls and lyse, releasing antigen into the gut or reticuloendothelial system; and (ii) cured *asd*$^-$ cells are completely avirulent due to the DAP requirement. Both types of attenuated *Salmonella* strains penetrate the gut and reach lymphoid tissue, although the *cya* and *crp* strains (4) appear to disseminate less than the *S. dublin* strain.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* LE392, *Salmonella dublin* SL1438 *strA aroA* (16), and plasmid pDB2300 (3) encoding a portion of the structural gene for *B. pertussis* FHA were described previously (12). *S. typhimurium* strains χ3710 and χ4072 were derived from mouse-virulent strain SR11 and were near isogenic with the following genotypes: *gyrA*1816, Δ*asdA-1*, Δ[*zhf-4::Tn10*], and *aroA*554 (χ3710); and *gyrA1816*, Δ*asdA-1*, Δ[*zhf-4::Tn10*], Δ*cya-1*, and Δ*crp-1* (χ4072). Plasmid M3007 was constructed by inserting a cloned *S. typhimurium asd* gene at the *Bgl*II site of pDB2300 (Fig. 1).

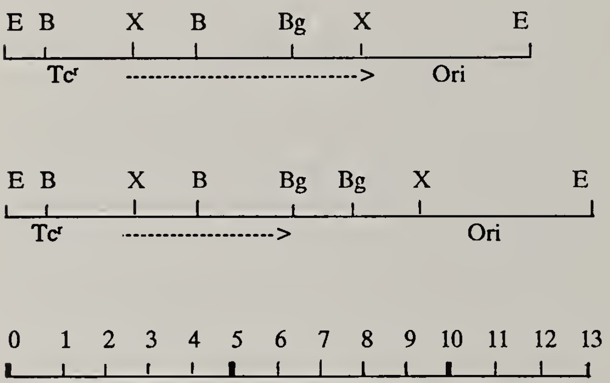

FIG. 1. Maps of plasmids pDB3200 and pM3007. Plasmids are shown in linear form; PDB2300 is at the top, pM3007 containing the *asd* gene is in the middle, and a scale in kilobase pairs is at the bottom. Presumed transcripts of *fhaB* are shown as dotted lines with arrows. Tc^r indicates the tetracycline resistance gene; Ori indicates the origin of replication. Restriction endonuclease abbreviations: E, *Eco* RI; B, *Bam* HI; X, *XhoI*; Bg, *Bgl* II.

Media. Bacteria were grown in LB (10) or other nutrient broth media as described (12), with tetracycline (15 µg/ml) for *S. dublin*. For LB plates, 15 g/liter of agar was added to LB broth. Sterile molten MacConkey agar base (Difco Laboratories, Detroit, Mich.) was supplemented with sterile 20% lactose to a final concentration of 1% lactose. Supplements included meso-DAP at 50 µg/ml, TC at 15 µg/ml, and nalidixic acid at 50 µg/ml as desired. Phosphate-buffered saline (PBS) contained the following, in g/liter distilled water: KCl, 0.20; KH_2PO_4, 0.20; Na_2HPO_4, 1.15; $MgSO_4$, 0.05; NaCl, 8.0; pH 7.0. Protease inhibitors (EDTA, 5 mM; phenyl-methane-sulfonyl fluoride, 2 mM; aprotinin, 0.05 U/ml) were added to PBS just before use for collection of gut wash and lung wash fluids (PBS-EPA).

Genetic manipulations. Similar techniques were used as before (10, 12), except that P22 transduction was used to construct the isogenic asd^- S. typhimurium strains (6).

Mouse inoculation. Bacteria were grown to mid-logarithmic growth stage in broth, centrifuged, and suspended in sterile PBS to a standard optical density to provide an oral dose of 2 to 5 x 10^8 CFU in 10 to 20 µl volumes for oral inoculation. Conventional BALB/c mice were not pretreated with antibiotics; they also were not anesthetized, unlike an earlier study (12). Mice were starved for food and water for 4 to 6 h, and fed the small inoculum from the plastic tip of an automatic pipettor.

Mouse experiments. Mice of both sexes (caged separately) were inoculated on day 0 in groups of 3 to 15 animals and caged in groups of 3 to 5 animals. For multiple inoculations, succeeding doses were at 3- or 4-day intervals after the first dose. At specific times postvaccination, three mice were chosen at random—usually one each from separate cages—anesthetized with ether, and exsanguinated by cutting the brachial artery. All samples from the three-mouse group were pooled, and thus represent the mean value for three animals. Blood was collected and serum was separated and stored at -20° C. For lung wash fluid collection, animals were immediately pertracheally cannulated with a blunt infant mouse feeding needle (Popper and Sons, New York), and up to 0.5 ml of ice-cold PBS-EPA was used to gently wash the lung. Samples were either discarded if any pink tinge was evident in the fluid (indicating contamination by blood) or were immediately frozen. For gut wash fluid collection, animals were dissected to reveal the intestine. A clamp was placed at the top of the duodenum, with another just above the intestinal area containing formed fecal pellets. This gut segment was dissected free, the end proximal to the cecum was placed in a disposable plastic tube, and 3 to 5 ml of ice-cold PBS-EPA was injected slowly via a 25-g needle inserted into the upper duodenum, gently flushing the fluid through the gut lumen. The collected fluid was centrifuged, and the supernatant was removed and frozen. Gut wash fluids were adjusted to a protein concentration of 50 µg/ml prior to immunoassay.

In some experiments, fecal pellets were collected from living mice and cultured quantitatively on MacConkey agar containing an appropriate antibiotic with or without DAP. Spleens were obtained from sacrificed mice, ho-

TABLE 1. Comparison of serum anti-FHA immunoglobulin elicitation by three live attenuated oral *Salmonella* vaccine strains expressing portions of *B. pertussis* FHA[a]

Group	*S. dublin*/pDB2300	*S. typhimurium* χ3710/pM3007	*S. typhimurium* χ4072/pM3007	P[b]
1 dose				
Day 5	0.046 (0)	0	0.226 (0)	NC
Day 8	0.056 (0)	0.139 (0.014)	0.137 (0.018)	NS
Day 14	0.124 (0)	0.294 (0)	0.530 (0.381)	NS
Day 20	0.052 (0)	0.372 (0.059)	0.608 (0.292)	NS
2 doses				
Day 6	0.012 (0)	0.115 (0.025)	0.222 (0.150)	NS
Day 13	0.181 (0)	0.584 (0)	0.688 (0.283)	NS
Day 21	0.469 (0.118)	0.826 (0.436)	1.448 (0.665)	NS
1 dose with TC, day 7	0.413 (0.191)	0.354 (0.072)	0.339 (0.186)	NS
No vaccine	0.098 (0)			

[a] Values are means of duplicate OD readings of anti-FHA immunoglobulin tested by ELISA on mouse serum pooled from 3 to 4 animals and diluted 1:50 (1:200).

[b] Probability that *S. typhimurium* χ3710/pM3007 and χ4072/pM3007 are different.

NC, Not calculated.

NS, not significant

TC, Tetracyline in drinking water.

mogenized in cold sterile PBS, and cultured quantitatively on LB agar with DAP.

Immunoassays. Enzyme-linked immunosorbent assays (ELISAs) performed against purified FHA bound to microtiter plates was as described previously (12). The purified FHA was a kind gift from Dr. Chun-nan Shih, Michigan State Department of Health. Total immunoglobulin was determined using a light chain-specific affinity-purified anti-mouse immunoglobulin second antibody. Immunoglobulin A-specific and IgM-specific anti-FHA titers were determined using second antibodies obtained from the same source (U.S. Biochemical Corporation, Cleveland, Ohio). Serum anti-FHA immunoglobulin determinations were reported, as they most sensitively detected the production of anti-FHA antibodies, and because anti-FHA IgA was not always detected.

RESULTS

Rationale. We wanted to determine whether the method developed by Nakayama et al. (13) to stabilize plasmids bearing foreign genes in attenuated *Salmonella* oral vaccine strains would be effective for plasmids encoding *B. pertussis* FHA protein. Because the *asd* gene product is required for synthesis of DAP, and DAP is required for complete peptidoglycan, bacteria which are *asd*$^-$ lyse unless supplied with DAP. Since DAP is an unusual amino acid essentially limited to bacterial peptidoglycan, it is not found in animal tissue. Thus, when the *asd* gene is carried by a plasmid in *asd*$^-$ *Salmonella* strains, strong selective pressure is exerted for plasmid maintenance. Additionally, such *asd*$^-$ bacteria-*asd*$^+$ plasmid "balanced lethal" vector systems may provide for effective delivery of protein antigens synthesized by the cells. As cells are spontaneously cured of plasmids in the body, bacterial lysis occurs, releasing antigens extracellularly. Since *S. typhimurium* cells are taken up by macrophages and undergo antigen processing, effective delivery of antigens may also occur due to antigen presentation by macrophages to lymphoid cells.

Comparison of three attenuated strains of *Salmonella* for anti-FHA antibody production. *S. dublin* and the two near-isogenic attenuated strains of *S. typhimurium* were compared for serum immunoglobulin (Table 1). *S. dublin* was a control, since the plasmid it contained

TABLE 2. Serum total immunoglobulin anti-FHA responses to live attenuated *S. typhimurium* χ4072/pM3007 as related to oral dose

Dose	ELISA OD at dilutions of 1:20 (1:50) on days:			
(CFU)	7	14	21	28
2 doses at 10^5	0	0	0.007 (0)	0
2 doses at 10^6	0	0.104 (0.058)	0.044 (0.018)	0.112 (0.086)
2 doses at 10^7	0.043 (0.018)	0.147 (0.102)[a]	0.173 (0.125)[a]	0.156 (0.128)[a]
2 doses at 10^8	0.055 (0.042)	0.142 (0.096)	0.248 (0.206)[b]	0.217 (0.216)[b]
3 doses at 10^7	ND	ND	0.325 (0.268)[b]	ND
3 doses at 10^8	0.100 (0.062)[a]	ND	ND	0.270 (0.200)[b]
No vaccine	0.003 (0)			

Numbers refer to mean values of duplicate determinations of ELISA OD at dilutions of 1:20 (1:50) on pooled serum from 3 to 4 animals.

[a] Values are repeatable, and 1:50 dilutions are ≥0.1 OD unit above background, indicating probable difference from the no vaccine control.

[b] Values are ≥0.2 OD units above no vaccine control even at a 1:50 dilution and are thus considered significantly different from no vaccine.

ND, Not determined.

was slightly different from that in *S. typhimurium,* thereby not permitting direct comparison to these latter strains. Immunoglobulin M and IgA analyses of these sera revealed that anti-FHA IgM was present at early time points for the *S. typhimurium* strains and then declined. Immunoglobulin A appeared to be present but in quantities lower than total immunoglobulin, and statistical significance was not achieved. Neither the IgM nor IgA data add to the conclusions which can be inferred from the single experiment total immunoglobulin results (Table 1).

The experiments were preliminary because only small numbers of mice were examined. Note that a control group with TC selection for plasmid maintenance was tested (as in earlier *S. dublin* studies; 12), and immunoglobulin responses were detectable in this control group, especially for *S. dublin.* For *S. dublin,* oral TC treatment or two doses of vaccine was necessary for induction of detectable antibody responses. However, both the *S. typhimurium* strains induced antibody without TC treatment.

The experiment results also suggested that two doses of the recombinant vaccine strains elicited higher antibody titers than a single dose. We did not test enough animals to show that the antibody levels for any group of three mice differed significantly between the two *S. typhimurium* vectors. Nevertheless, the double deletion mutant χ4072 showed higher antibody titers for each of the six groups tested without TC. We interpret our data to mean that the *cya-crp* mutant was at least as good as *aroA* mutants for delivering FHA antigen in this system.

Assays for anti-FHA specific IgA in gut wash fluids gave low and inconsistent ELISA titers in this experiment. For lung washes, occasional samples demonstrated low ELISA titers. Thus, it cannot be concluded that secretory IgA was induced by immunization with either *S. typhimurium* strain. We did not detect much *S. dublin*-induced anti-FHA IgA in this experiment (data not shown), although we found such IgA in previous oral immunizations when mice were pretreated with streptomycin prior to vaccination (12).

Curing of recombinant plasmids in vivo. More than 200 individual colonies each of *S. typhimurium* χ3710 and χ4072 were recovered from culture of fecal pellets on MacConkey agar plus DAP. Upon testing, none of the colonies required DAP for growth. In similar previous experiments for recombinant *S. dublin* SL1438 (12), as well as in this study, fecal pellet cultures on various days revealed that 2 to 100% of colonies recovered on drug-free medium were sensitive to TC, indicating rapid plasmid curing.

Dissemination of *Salmonella* vectors to the spleen. As a measurement of ability to invade tissue beyond the gut, we measured the numbers of colonies per vaccinated

TABLE 3. Anti-FHA immunoglobulin in various samples from mice immunized orally with *S. typhimurium* χ4072/pM3007[a]

Treatment	OD at dilution of 1:20 (1:50)		
	Serum	Gut wash	Lung wash
2 doses[b]	0.274 (0.224)[c]	0 (0)[d]	0.046 (0.018)[d]
3 doses	0.248 (0.176)[c]	0.007 (0)[d]	0.021 (0)[d]
Normal mice	0.003 (0)	0	0

[a] Numbers refer to mean values of duplicate determinations of ELISA OD at dilutions of 1:20 (1:50) on pooled samples from three to five mice per group tested 7 days after last oral dose.

[b] ca 2 x 10^8 CFU/dose; doses were 3 or 4 days apart.

[c] Serum immunoglobulin values exceed control by $\geq$0.2 OD units and are significantly different from controls.

[d] Values are not significantly different from controls.

mouse spleen. Animals vaccinated orally with 2 to 5 x 10^8 CFU showed ranges of the following numbers of colonies in spleen on days 5 through 14 postvaccination: *S. dublin*, 10 to 138, median 76; *S. typhimurium* χ3710, 0 to 31, median 0; *S. typhimurium* χ4072, 0 to 1, median 0.

Oral vaccine dose and number of immunizations required for antibody response. Multiple doses of *S. typhimurium* χ4072 vaccine were given to mice and serum immunoglobulin data obtained (Table 2). Two doses at 10^7 CFU/mouse appeared to elicit an antibody response. Since a dose of 10^7 CFU is 20- to 50-fold lower than the doses used in previous experiments, it seems likely that multiple small doses of vaccine may elicit antibodies.

Anti-FHA antibody in serum or gut and lung wash fluids after two doses of oral vaccine. The immunoglobulin in serum, gut wash fluid, and lung wash fluid was quantitated for *S. typhimurium* χ4072 at a single time point (Table 3). The results of this experiment suggested that specific immunoglobulin may be found on mucosal surfaces, but the OD readings were low and were not statistically significant for these small groups.

DISCUSSION

Although parenteral vaccines will likely continue to play an important role in immunization against pertussis, especially if pertussis toxoid can be added to diphtheria and tetanus toxoids, a live attenuated oral vaccine may also be useful. Such a vaccine has the following advantages: (i) *Salmonella* species grow rapidly in simple medium; (ii) parenteral immunization is avoided; (iii) pertussis antigens need not be purified in the laboratory; (iv) pertussis antigens are delivered to the mucosa and to the reticuloendothelial system; (v) humoral and secretory antibodies are elicited and cellular immunity also may be elicited; (vi) the enteric capsule formulation used for the live attenuated typhoid vaccine is likely to deliver *S. typhimurium* successfully to the intestine (9); (vii) the technology to prepare oral vaccine strains can be completed in industrialized nations, while vaccine production using these strains can be accomplished inexpensively in poor nations; (viii) the same vaccine would immunize against salmonellosis; and (ix) several *B. pertussis* antigens could be included in a single vaccine strain.

The most striking finding of these experiments is the success of the "balanced lethal" vector system for attenuated oral vaccine strains of *Salmonella* in stabilizing recombinant plasmids. It is too early to say whether $aroA^-$, cya^- crp^-, or other attenuated *Salmonella* are superior for use in humans. However, the asd^- attenuated *Salmonella* strain carrying an asd^+ recombinant plasmid is clearly a superior method to deliver chimeric antigens to the gut, and neither the strains nor the plasmids need carry drug resistance genes. Selection for recombinants is accomplished merely by selecting for growth on bacteriological media lacking supplemental DAP.

The enhancement of antibody levels by multiple oral doses of vaccine is another important finding. One potential problem with live attenuated oral *Salmonella* vaccines has been the assumption that a single dose of vaccine would immunize against the *Salmonella* vector and prevent use of that strain again for antigen delivery. This assumption is clearly not true in our system in mice, and live oral human *S. typhi* vaccines are effective in multiple doses (9).

These studies are preliminary because the experiments were small, but they have been duplicated several times with similar results (although anti-FHA IgA is usually found in gut wash fluids). A new *asd*$^+$ plasmid is now being constructed that encodes several *B. pertussis* antigens, including part of the pertussis toxin gene. More rigorous studies will be performed once that plasmid has been introduced into appropriate attenuated vector strains. When ideal live oral attenuated pertussis vaccines of this type become available, they should be tested in intranasal animal infection models, because they may be anti-infective, as well as antitoxic. Only field trials in humans can delineate the efficacy of such vaccines to prevent human infection and disease.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI25055 from the National Institutes of Health to C. P. Additional support was provided by the University of Missouri in the form of 1 year of research leave for C.P.

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DISCUSSION

DR. SHAHIN: Have you tried any internasal challenges with *Bordetella* to see if you can increase clearance from the lungs?

DR. PARKER: We have not. We have not wanted to work extensively in animals with a strain containing the tetracycline resistance gene and so we are trying to construct a plasmid that is entirely free of antibiotic resistance markers, etc., and perhaps we will also make some pertussis toxoid antigen before we do more extensive animal work.

DR. DE MAGISTRIS: How do you explain that you did not get IgA antibodies by immunizing with this construct? Are you going to change the way to immunize to get an IgA response?

DR. PARKER: We did not measure IgA in all cases. We did not have enough sample in all cases from this experiment. But using the *Salmonella dublin* vector that is *aroA*, we have shown, in a recently published paper that we can find gut IgA in significant amounts. Of course, in spite of what the immunologists write, it is hard for me to understand if that means that there will be sufficient antibody or cell mediated immunity or whatever on the mucosal surface in the lung and nasopharynx. I do not know that yet.

DR. SHAHIN: I just wanted to make a brief comment on that point. I actually think that you have got it and you do not know it. You were diluting your secretions 1 to 20 or 1 to 50 and if you look at people like John Nedrud and John Eldridge, who publish on this sort of thing, you really need to have very high concentrations.

DR. PARKER: I am sorry, I gave you a wrong impression. Our secretions were concentrated to give a constant protein reading. They were not diluted. They were concentrated.

Analysis of Protective Mucosal Immunity to Respiratory *Bordetella pertussis* Infection

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Pertussis is a disease of the respiratory tract in humans, typified by bouts of paroxysmal coughing and caused by the gram-negative pathogen *Bordetella pertussis*. *B. pertussis* is transmitted by aerosol droplets and exhibits a tropism for the cilia of the respiratory epithelium; however, the infection does not progress to bacteremia, but remains localized in the airways and lungs (7).

In autopsies of children who died of uncomplicated pertussis pneumonia (with no other apparent respiratory infection), masses of gram-negative bacilli have been described between the cilia of the epithelium lining the trachea and bronchial tree; these bacteria were presumably *B. pertussis* (12). Concurrently, several of these specimens demonstrated a shedding of the ciliated epithelial cells lining the respiratory tract. The ability to culture *B. pertussis* from the nasopharynx early in infection, coupled with the observation of masses of bacteria enmeshed in the cilia of the bronchial tree, suggests that adhesion of *B. pertussis* to cilia is a critical step in the bacteria's success as a pathogen.

Pertussis is an endemic disease that occurs in epidemics worldwide with a periodicity of approximately 3 years (8). Widespread vaccination using a parenteral vaccine of whole killed *B. pertussis* has been successful in controlling the incidence of disease both in the U.S. and abroad (6, 7).

While the currently used whole-cell pertussis vaccine is effective in controlling disease, it falls short of being an ideal vaccine in that it is reactogenic, fails to confer lasting immunity, cannot fully protect those at greatest risk (i.e., infants younger than 6 months), and probably does not prevent infection or decrease disease transmission (6, 8). Furthermore, the mechanism of protective immunity elicited by the whole-cell vaccine remains unknown.

Two preparations of acellular pertussis vaccines, composed of either pertussis toxoid or pertussis toxoid and filamentous hemagglutinin (FHA) were demonstrated to protect against severe disease in a double-blind controlled trial conducted in Sweden (1). However, protection from disease in this trial did not correlate with serum antibody to either pertussis toxin or FHA. The ability of pertussis toxoid and FHA to provide clinical protection in the absence of a serum antibody correlate of immunity illustrates the current lack of understanding of the immunologic basis of protective immunity to pertussis.

One impediment to this understanding of the infectious process and protective immunity to pertussis is that pertussis is solely a human disease: as such, there is no satisfactory animal model that reflects all aspects of *B. pertussis* pathogenesis. However, certain features of the human disease process are seen in a mouse aerosol challenge model (18). These features include specific attachment of *B. pertussis* to the ciliated epithelium of the respiratory tract, leukocytosis, and an age-dependent severity of disease (18). While not a model of whooping cough per se, the mouse model does reflect key steps in pathogenesis, i.e., specific attachment and colonization, susceptibility to the action of bacterial toxins, and inflammation induced by gram-negative infection.

Several novel aspects of pertussis immunity are currently being analyzed, in vivo and in vitro in humans as well as in mouse models of respiratory infection. T-cell immunity to antigens of *B. pertussis* is discussed in this volume by DeMagistris, Mills, and Blumberg.

Another area beginning to be explored is that of local immunity to pertussis. Several groups have observed that following pertussis infection, immunoglobulin A (IgA) to pertussis antigens can be detected in the serum and saliva (9, 11, 23). Epidemiologic studies indicate that, unlike parenteral whole-cell pertussis vaccination, naturally occurring pertussis infection confers long-lasting protection from reinfection (10). Thus, natural infection confers lasting protective immunity at the same time that a mucosal immune response to antigens of pertussis is observed.

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Natural infection with *B. pertussis* may confer lifelong immunity because it may elicit a potent protective immune response in the respiratory mucosa. Since 1926, it has been appreciated that a protective respiratory immune response can occur in the absence of a serum IgG response to the respiratory pathogen (5). Thomas (22) has demonstrated that administration of an aerosol of killed whole pertussis vaccine to adult volunteers induces increases in pertussis antibodies in nasal secretions but not in serum. Intramuscular administration of the same vaccine causes increases in pertussis antibodies in serum but not in nasal secretions (22). Aerosol administration of genetically attenuated, live *B. pertussis* to mice was shown to prevent colonization following subsequent aerosol challenge with virulent *B. pertussis* (16).

This leads one to question the role of the respiratory mucosa in immunity to pertussis. The nasal mucosa and bronchial tree contain specialized mucosal follicles — the nasopharyngeal tonsils and bronchus-associated lymphoid tissues — that consist of aggregates of lymphocytes and antigen-presenting cells, analogous to the Peyer's patches of the gut (3, 4). Overlying these aggregates is a layer of microfold epithelium, a highly endocytic cell that functions to transcytose antigens from the lumenal surface to the basolateral surface of the epithelium (14). Antigens exocytosed from the basolateral membrane of the epithelium are taken up by antigen-presenting cells and lymphocytes of the mucosal follicle. These mucosal follicles, therefore, are sites of active uptake of antigens present at mucosal surfaces. Lymphocytes stimulated by antigens in the follicle begin to divide and egress via the draining lymphatics of the gut, transit the mesenteric lymph nodes, and enter the blood circulation at the thoracic duct. Recirculating lymphocytes can leave the circulation at particular sites in the blood vessels by interacting with specialized high columnar endothelial cells; this interaction allows mucosally stimulated lymphocytes to egress from the circulation and enter the lamina propria of mucosal tissues, where they can terminally differentiate into antibody-secreting plasma cells.

The present study is an analysis of the B-cell memory response in the respiratory tract following either immunization or immunization and infection with *B. pertussis*.

MATERIALS AND METHODS

BALB/cAnNcR mice were obtained, with mothers, on day 3 postpartum from the Animal Production Program, Division of Cancer Treatment, National Cancer Institute, Frederick, Md. Mice were immunized intraperitoneally on days 5 and 12 postpartum with glutaraldehyde-inactivated pertussis toxin prepared in this laboratory as previously described (2).

Aerosol challenge. A 21-h culture of *B. pertussis* 18323 grown on Bordet-Gengou agar was suspended in sterile phosphate-buffered saline at a concentration of approximately 10^9 CFU per ml inoculum. The challenge inoculum was administered to mice as an aerosol using a standard nebulizer (Fisons Corp., Bedford, Mass.) within a biosafety level 3 glove box (Blickman Co., Weehawken, N.J.) as previously described (18, 20).

Analysis of respiratory and serum immunoglobulin. Mice anesthetized with 2,2,2-tribromoethanol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) were bled from the brachial artery, and their tracheas were cannulated with a piece of PE-50 polyethylene tubing (Clay Adams, Parsippany, N.J.). Sterile phosphate-buffered saline (0.3 ml) was gently instilled into the lungs and withdrawn three times. The bronchoalveolar lavage fluid was centrifuged and the supernatant removed and frozen at -20°C prior to analysis.

Serum and bronchoalveolar lavage fluid were analyzed for specific antibody to pertussis toxin by an enzyme-linked immunosorbent assay as previously described (13, 20). Total anti-pertussis toxin titers are expressed as the reciprocal of the endpoint dilution, calculated by extrapolation to zero from the linear part of the titration curve.

Limiting dilution analysis of memory B lymphocytes. A limiting dilution adoptive transfer assay system was used to enumerate the number of pertussis-toxoid-stimulable B lymphocytes from the lungs and spleens of mice that had been immunized with 8 μg of pertussis toxoid on days 5 and 12 postpartum or immunized on days 5 and 12 postpartum and aerosol-challenged on day 19 postpartum (17). Nylon-wool-purified T cells from pertussis-toxoid-immunized mice were used as a source of T-cell help. Organ cultures were stimulated with 10^{-8} M glutaraldehyde-inactivated pertussis toxoid in vitro, and culture supernatants were scored on pertussis-toxin-coated plates by enzyme-linked immunosorbent assay.

TABLE 1. Antipertussis antibody in serum and bronchoalveolar lavage

	Serum		Bronchoalveolar lavage fluid	
	Titer[a]	Isotype	Titer[a]	Isotype
Unimmunized + uninfected	<50	ND	<20	ND
Immunized + uninfected[b]	3,200	IgG	<20	ND
Immunized + infected[c]	13,000	IgG + IgA	500	IgG + IgA

[a] Mean reciprocal titer.
[b] Sampled 7 days after last immunization with pertussis toxoid.
[c] Sampled 21 days after respiratory infection.
ND, Not done.

RESULTS AND DISCUSSION

Local respiratory and serum antibody response to immunization and aerosol infection. It has been previously demonstrated that neonatal mice administered a primary and secondary intraperitoneal injection of 8 μg pertussis toxoid are protected against leukocytosis, weight loss, and death following aerosol challenge with a lethal inoculum of *B. pertussis* 18323 (19, 21). To dissect components of protective immunity in this model, the titers of serum and respiratory anti-pertussis toxin in young mice were evaluated following intraperitoneal immunization with pertussis toxoid or after immunization with pertussis toxoid and aerosol challenge with *B. pertussis* 18323. After immunization with pertussis toxoid, significant titers of serum IgG anti-pertussis toxin were detected, in the absence of serum IgA anti-pertussis toxin as well as in the absence of detectable respiratory antibody (Table 1). Following immunization and *B. pertussis* aerosol challenge, serum anti-pertussis toxin titers increased further, and both IgG and IgA anti-pertussis toxin antibodies were detected in sera as well as in the bronchoalveolar lavage fluids of immunized and infected animals.

TABLE 2. Frequency analysis of pertussis toxin memory B lymphocytes

	Hilar lymph nodes	Lungs	Spleen
Unimmunized + infected	<37[a]	<16[b]	1
Immunized + uninfected[c]	ND	104	17
Immunized + infected[d]	59	342	85

[a] 0 clones per 2 x 10^6 input cells.
[b] 0 clones per 6.5 x 10^6 input cells.
[c] Sampled 7 days after last immunization with pertussis toxoid.
[d] Sampled 21 days after respiratory infection.
ND, Not done.

Frequency analysis of pertussis-toxin-specific B cells. A limiting dilution adoptive transfer assay system was used to determine the frequency of pertussis-toxoid-specific memory B lymphocytes isolated from the lungs, draining lymph nodes of the lungs and spleens of mice that had been either immunized intraperitoneally on days 5 and 12 postpartum or immunized and infected with an aerosol of *B. pertussis* 18323. Anderson and co-workers have shown that antigens in the peritoneal cavity can be delivered to the bronchus-associated lymphoid tissue of the lungs by macrophages that traffic along lymphatic channels of the diaphragm from the peritoneum to the lungs (15). Therefore, there is a mucosal as well as a systemic component of intraperitoneal immunization. This feature of intraperitoneal immunization has been taken advantage of in analyzing memory B-cell populations in the respiratory mucosa either after immunization or after immunization and respiratory challenge. There was an increase in the number of pertussis-toxoid-reactive B lymphocytes isolated from the lungs and spleens of immunized mice in comparison with unimmunized controls (Table 2). These pertussis-toxin-specific B-cell populations displayed an even further increase in immunized mice that had survived a respiratory infection with *B. pertussis*. An increase in number of pertussis-toxoid-reactive B lymphocytes was also detected in the draining lymph nodes of the lungs of immunized and infected mice in comparison with unimmunized, uninfected controls.

Isotype analysis of the supernatants from pertussis-toxin-specific B-cell clones revealed a greater proportion of clones making IgG relative to IgA in the lungs and IgG and IgM relative to IgA in the spleens of immunized animals (Table 3). Clones from immunized animals that

TABLE 3. Isotype analysis of clones derived from pertussis toxin memory B lymphocytes

	No. of clones	% clones expressing:						
		M	G	A	M + G	M + A	G + A	M + G + A
Immunized lungs	3	0	100	0	0	0	0	0
Immunized + infected lungs	22	0	23	0	0	0	36	41
Control spleen	1	100	0	0	0	0	0	0
Immunized spleen	23	30	52	0	4	4	4	4
Immunized + infected spleen	27	0	81	0	0	0	19	0

had been infected with an aerosol of *B. pertussis* displayed a majority of clones from the lungs, giving rise to IgG and IgA production; however, the majority of clones derived from the spleens of these same mice produced IgG.

Since intraperitoneal immunization of neonatal mice is sufficient to protect against lethal *B. pertussis* aerosol challenge, the finding that immunized mice have detectable serum anti-pertussis toxin, but no detectable respiratory antibody, suggests that high titers of toxin-neutralizing antibodies are not required in the lungs at the time of respiratory infection. This hypothesis in turn suggests that either (i) serum antibody alone (perhaps along with low levels of antibody in the lung that would not be detected in the assay) is sufficient to protect mice at the time of *B. pertussis* aerosol challenge or (ii) intraperitoneal immunization leads to the dissemination of toxin-specific memory-B lymphocytes to the respiratory mucosa.

In addition to these two nonexclusive possibilities, other non-B-cell-mediated protective mechanisms may come into play. It has been found that intraperitoneal immunization with pertussis toxoid disseminates a primed population of memory-B lymphocytes to the respiratory mucosa. Significant IgA production was seen after immunization and infection, but not after immunization alone. Infection in the absence of immunization is not sufficient to elicit either respiratory or serum IgG or IgA antibody responses to pertussis antigens (data not shown). Successful prophylactic vaccination depends on eliciting a long-lived protective memory-B- and T-cell response at the site of infection, as high levels of neutralizing antibodies or effector T lymphocytes cannot be maintained over long periods of time in the absence of constant restimulation with antigen. Primed memory lymphocyte populations may be stimulated to produce protective antibody or effector T lymphocytes locally at the time of natural infection by exposure to the immunizing antigen on the pathogen. While the data presented here reflect B-cell-mediated immunity, the principles of disseminating antigen-specific memory lymphocyte populations to the site of infection — in the case of pertussis, the respiratory mucosa — applies equally well to T cells. Greater understanding of the mechanism or mechanisms of protective immunity to *B. pertussis* infection will aid in the design of optimal vaccines that provide durable immunity.

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DISCUSSION

PARTICIPANT: Can you tell me about what percentage exist in the stomach?

DR. SHAHIN: They apparently make it. John Eldridge's experiments are all with oral feeding. They are not injected intraduodenally. These are the data he has used to show that not only do they make it across the patch into the macrophages in the patch but that they elicit an IgA response so they are stable enough. I cannot tell you about what percentage is lost in the stomach, but if you compare it to antigen that is not encapsulated, it is much, much better.

DR. PARKER: I might point out that one of the advantages of the *Salmonella* method is that the *Salmonella* do still penetrate to the reticuloendothelial system, and so they deliver the antigen very efficiently. Of course, there are certainly advantages in having something that is relatively inert and cannot possibly harm the host. I wish we could say that that were true about both of these things but I am very excited by your results.

DR. SHAHIN: I think there is clearly benefit to not only both systems but to looking at other things like adenovirus and other viruses as oral vectors. Certainly, molecular genetics offers us a great experimental research tool because the cost of purifying an antigen in quantity to do these kinds of experiments is exorbitant. Not only that, I think you can have different applications that are equally useful so I do not think one excludes the other. We need all the information we can get. As you can see from today's session, not only is the disease complicated, the immune system is complicated.

DR. PARKER: I was going to add that I once heard a fairly famous person define an immunologist as a person who does experiments in which the mouse does the work and a bacteriologist as someone who lets the bacteria do the work, and so I must be in the latter and you in the former.

DR. DE MAGISTRIS: Did you look at the Ig isotype produced by the lymphocytes recovered from the lung?

DR. SHAHIN: That is a really good question. We have not done it in these experiments but in other experiments that will be included in the symposium manuscript. We have looked at pertussis toxoid specific precursors in the lung and we have done a little bit of FHA in a different adoptive transfer system that allows us to isotype. We see both IgG and IgA produced in the lung. They are both there, so there is a local component of the IgG response in the lung and what we would like to do is sort out what effective functions are required in the lung to get protection.

DR. CHANDLER: Have you tried the experiment where you primed first intraduodenally and then boosted intranasally?

DR. SHAHIN: We have not done that yet. I know what you are talking about. Up until these experiments you have always had to prime intraduodenally and then boost intranasally to see anything in the lungs at all. This is the work of John Nedrud with Sendi. We have not tried that because we really wanted to shoot for the oral and then the intranasal was really to characterize the pure system. We can do that eventually but we would really like to see if we can work towards just oral immunization.

DR. STAINER: A couple of practical questions. I was surprised to see the use of the word alum in your slide, number one. I want to know what that meant. Was it alum? Was it aluminum phosphate, aluminum hydroxide or ferric alum or potassium alum or which of those chemicals was it? Secondly, have you done any in vitro experiments with these microspheres, the encapsulated antigens to see whether with enzymes or pH studies the antigen is still there; the FHA is still there, how it is released and things like that. Have you done anything at all in that area?

DR. SHAHIN: The first is an immunologist's mistake. We think everything is alum as long as it has got aluminum in it. It was aluminum hydroxide. It was actually alhydrogel to be exact. The second question is something that actually has been done much more by my colleagues but all the batches of microspheres are tested in vitro, if you will, to determine their FHA content. That is how I can tell you that the dose I gave contained 25 micrograms of antigen for instance. In terms of chemical properties, you would have to talk to Rick Gilley who I believe is in the audience.

Investigation of the Role of the *Bordetella pertussis* Virulence Factors in Disease

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It is generally agreed that whooping cough is the result of systemic intoxication by pertussis toxin (PT). However, the goal of producing a safe, effective, defined component pertussis vaccine has remained elusive. The whole-cell vaccine, containing every possible antigenic component produced by the bacterium, confers the desired level of protection but at the expense of safety. Two first-generation acellular vaccines consisting of toxoided PT alone or in combination with filamentous hemagglutinin (FHA) appear to be safe but do not confer the desired level of immunity (1). This circumstance suggests that additional antigenic components are required. To design the ideal vaccine, the antigens required for protection, which are presumably some subset of the whole-cell vaccine, must be identified; also, the source of the adverse effects must be both identified and eliminated.

This research was designed to complement studies evaluating individual proteins as protective antigens by attempting to understand the role played by various virulence factors in the disease process. It was assumed that challenging an animal with a bacterial mutant unable to synthesize a single virulence factor is somewhat analogous to immunizing the animal with a single protein and then infecting it with a wild-type strain. This analogy works well when neutralization of an activity is required for protection, but breaks down when opsonizing antibodies are induced, since these antigens could be irrelevant to the disease process but important for protection. However, useful information can be obtained by this approach. Previous studies have shown that *Bordetella pertussis* mutants that only fail to produce FHA or dermonecrotic toxin (7) are unaltered in virulence, whereas mutants that fail to produce adenylate cyclase (AC) toxin or PT are severely deficient in their ability to cause a lethal infection in infant mice challenged intranasally (7, 9). In this study, the researchers monitored the proliferation of the wild-type and mutant bacteria in the lungs of the infected animal and examined the pathological changes induced by the presence of the bacteria in the respiratory tract. The results suggest that two toxins, PT and AC toxin, play essential but quite distinct roles in promoting the disease process and that both should be assessed as potential antigens in an acellular vaccine.

MATERIALS AND METHODS

Infant mouse studies. Six-day-old BALB/cByJ mice (Jackson Laboratory, Bar Harbor, Maine) were infected intranasally as in previous studies (7) and sacrificed by cervical dislocation or inhalation of methoxyflurane or carbon dioxide. In quantitative studies, the lungs were removed and colony counts determined as previously reported (T. S. M. Goodwin and A. A. Weiss, Infect. Immun., in press) by suspending the lungs in 5 ml Stainer-Scholte broth, disrupting for 60 sec in a Model 80 Stomacher lab-blender (Seward Medical, London, England), and plating various dilutions on Bordet-Gengou agar.

Histological studies. The mice were humanely killed. The lungs, along with the entire respiratory tract, heart, and upper digestive tract, were removed from each body. The lungs were fixed by tracheal infusion of 10% neutral-buffered formalin sufficient to restore normal distension (approximately 0.5) followed by immediate emersion in the fixative. Formalin-fixed lungs were trimmed and processed routinely for paraffin embedding. Paraffin-embedded tissues were sectioned at 6 μm and stained with hematoxylin and eosin. Selected specimens were stained with giemsa and by the Brown and Brenn method for staining gram-negative and -positive bacteria.

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RESULTS

Virulence of the mutants in the infant mouse model. Intranasal challenge of 6-day-old BALB/cByJ mice was previously performed; the results from several of these studies are summarized in Table 1. The virulent-phase parental strain, BP338, was able to cause a lethal infection at a dose of 2 x 10^4, whereas BP347, an avirulent mutant, was unable to cause a lethal infection at a dose of 10^7. Mutants deficient in a single virulence factor were also examined: two mutants, BP348 (deficient in AC toxin production) and BP357 (deficient in PT production), were severely diminished in their ability to cause lethal infection. Mutants deficient in FHA (BPM409 and BPM1821) were not impaired in their ability to cause a lethal infection. A mutant deficient in dermonecrotic toxin production (BPM1809) was tested. Unexpectedly, this mutant was unimpaired in its ability to cause lethal infection, suggesting that this toxin does not contribute significantly to the disease process.

The ability of several mutants deficient in the expression of *vir*-regulated genes of unknown phenotype to cause a lethal infection was also examined. Only two of the mutants of unknown phenotype, BPM2041 and BPM3171, were affected in their ability to cause lethal infection, requiring a tenfold greater challenge dose than the wild type (Table 1). Nothing more is known about these mutants except that (i) BPM2041 is deficient in the ability to invade HeLa cells (3), and (ii) BPM3171 maps about 3 kilobases downstream from the pertussis toxin gene (10).

TABLE 1. Median lethal dose for bacterial strains

Strain	Relevant phenotype (reference)	LD_{50}
BP338	Virulent (7)	<1.5 x 10^4
BP338-5	BP338, fimbria 2 minus (9)	≤ wild type
BP347	Avirulent phase (7)	>1.0 x 10^7
BP348	AC minus (7)	≈10^6
BP357	PT deficient (7)	>10^6
BP357	BP357, fimbria 2 positive (7)	>10^6
BPM409	FHA minus [*fhaB-1*::Tn*5 lac*] (7)	≤ wild type
BPM1821	FHA minus [*fhaA-1*::Tn*5 lac*] (7)	≤ wild type
BPM1809	Dermonecrotic toxin minus (7)	≤ wild type
BPM2041	Unknown *vir*-regulated gene (7)	>10^4, <10^5
BPM3171	Unknown *vir*-regulated gene (7)	≈2.7 x 10^5
BPM177	Unknown *vir*-regulated gene (7)	≤ wild type
BPM245	Unknown *vir*-regulated gene (7)	≤ wild type
BPM1579	Unknown *vir*-regulated gene (7)	≤ wild type
BPM2055	Unknown *vir*-regulated gene (7)	≤ wild type
BPM2119	Unknown *vir*-regulated gene (7)	≤ wild type
BPM2123	Unknown *vir*-regulated gene (7)	≤ wild type
BPM2859	Unknown *vir*-regulated gene (7)	≤ wild type

Isolation of bacteria from the lungs. Proliferation of *B. pertussis* in the lungs of infant mice challenged intranasally was examined (Goodwin and Weiss, in press). A sublethal dose of wild-type strain (BP338) rapidly proliferated in the lungs of the mice, starting with 500 cells at the time of challenge to 10^7 cells at day 15 (Fig. 1). The infection cleared in about 40 days. In contrast, no bacteria were detected in the lungs 48 h later when the mice were challenged with 10^7 of an avirulent phase mutant (BP347) which is unable to express any of the virulence regulated genes (data not shown). This demonstrates that failure to express the virulence-regulated genes results in rapid-elimination of the bacteria from the respiratory tract.

Experiments were performed with mutants deficient in one of the virulence factors with initial challenge doses of 500 bacteria. Nearly identical results to those obtained with the wild-type strain were seen with the FHA mutant (BPM409). The PT deficient mutant BP357 also gave a similar profile; however, the number of bacteria recovered was slightly reduced, especially after 1 wk. Thus, PT apparently is not essential for initial bacterial growth in the lungs, but appears to play a role at later stages in the infection. In contrast, the AC toxin mutant BP348 were used was rapidly cleared from the lungs with no viable bacteria remaining 10 days post-challenge, suggesting that AC toxin is required for the bacteria to initiate the infection. A different result was obtained when the mice were challenged with a high dose (10^5 bacteria) of the AC mutant BP348 (Fig. 1). Instead of being rapidly cleared from the lungs, a persistent infection was set up that lasted even longer than the 40 days seen with the wild-type strain.

Studies of mixed infections. Mixed infections with mutants BP357 and BP348 were used to determine if the PT produced by BP348 and the AC toxin produced by BP357 were sufficient to restore virulence. This mixture was not able to cause a lethal infection at the same dose as could the wild-type strain (7), suggesting that the production of the two toxins by two different strains was not equivalent to the production of both toxins by a single cell. The inability to cause lethal infection was not due to the inability of either of the mutants to persist, however, since both strains were isolated from the lungs of the mice. Recent studies have determined that the bacterial recoveries from the mixed infections were similar in total numbers to those from the wild-type infection, with the PT mutant (BP357) always comprising the majority of the bacteria recovered. The recovery of the AC toxin mutant (BP348) varied enormously, suggesting that the presence of the PT mutant sometimes aided the survival of the AC toxin mutant.

Histological studies. The pathological changes induced by the infection in the lungs were investigated. The mice were challenged with the bacteria and sacrificed on various days post-infection. The lungs were removed, fixed, sectioned, and stained. For comparison, Fig. 1 indicates the dates the mice were sacrificed together with the colony count recoveries from other studies. A mild to moderate multifocal pneumonia was observed 12 days after challenge with approximately 10^3 cells of strain BP338 (a sublethal dose). When challenged with a lethal dose of about 10^4 bacteria, a severe pneumonia was observed 15 days post-infection. Similar, although milder, pathology was observed 15 days post-infection with the AC mutant (BP348) at a sublethal challenge dose of 10^6. This finding suggests that the AC mutant is capable of eliciting an inflammatory response and causing pathological changes in the lungs of the infected animal, but is insufficient to result in the death of the animal. Studies are under way to determine if the different mutants generate different pathological changes during the course of the infection.

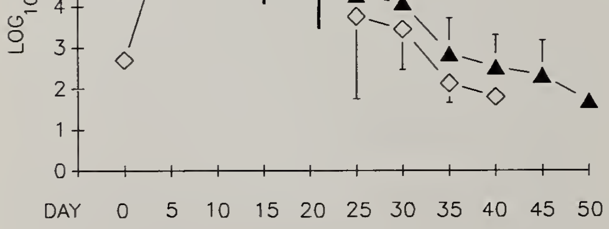

FIG. 1. Recovery of bacteria from the lungs of mice. The mice were infected with approximately 500 BP338 (wild type) or approximately 10^5 BP348 (AC-toxin deficient). The geometric mean and standard deviation of at least three mice per data point are plotted. The hatched lines denote the area below the limit of detection, or 50 bacteria per lung. The arrows indicate the times when the mice were sacrificed for histological studies. Downward arrows indicate BP338; upward arrows indicate BP348.

DISCUSSION

The intranasal challenge of infant mice has proven to be a very useful model for human whooping cough. As in whooping cough, the infection is lethal for infants but not for older individuals; also, the infection causes lymphocytosis and other signs and symptoms seen in human disease to develop in the mice (5). Mice readily develop *Bordetella* pneumonia which, although not well-documented in humans, is in fact a common occurrence as suggested by the older literature (8). By investigating the nature of the lethal infection and the ability of the bacteria to proliferate in the lungs of infant mice, researchers are beginning to gather some insights into the nature of human whooping cough and the roles of the various virulence factors in promoting the disease.

In the present studies, two toxins appear to be critical for the ability of the bacteria to cause a lethal infection: PT and AC toxin. These toxins appear to be required at different stages in the disease process and probably perform quite different functions. PT does not appear to be required for initial growth in the lungs, but could be responsible for the lethal effect, since the course of the infection is similar to wild type when bacterial numbers in the lungs are compared, but radically different when lethal outcome is compared. PT also has been shown to be a protective antigen in mouse studies (6), and this study's results would suggest that neutralizing antibodies to PT would be important for protection from disease.

The AC toxin has not been evaluated as a protective antigen because purified material has not been available until recently. The present studies using the AC toxin mutant are intriguing. At low challenge doses, this factor appears to be essential for survival and colonization of the respiratory tract. In vitro assays have demonstrated that the toxin is a potent inhibitor of immune cell function (11); this would suggest that elaboration of this toxin would poison the immune response and permit the bacteria to grow unmolested in the respiratory tract.

Two virulence factors that do not appear to be required for the lethal infection of infant mice are dermonecrotic toxin and FHA. Very little is known about dermonecrotic toxin except that it appears to act by inducing vasoconstriction (2); its role as a protective antigen has not been evaluated. Kimura et al. (4) have shown that FHA is a protective antigen for adult mice as determined by reduced lung colonization after immunization with FHA. However, the present studies have shown that failure to produce FHA does not appear to change the rate of bacterial growth in the lungs of infant mice. Combining these two sets of findings suggests that FHA is a protective antigen probably because it reduces the bacterial load by inducing opsonizing antibodies, and that neutralization is not required for protection.

These studies have important vaccine implications. Vaccines consisting of PT and FHA do not prevent infection by the bacteria, but may result in a less serious course of disease (1). The untested implications of AC mutant results is that a neutralizing response to this toxin could prevent both infection and disease. Since *B. pertussis* is exclusively a human pathogen, elimination of the reservoir of infected people could eradicate the disease, as was successfully accomplished with smallpox. The availability of purified AC toxin should allow these issues to be addressed in future studies.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-23695 from the National Institute of Allergy and Infectious Diseases and by a Pew Foundation Fellowship to A.A.W.

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DISCUSSION

DR. HANSKI: Did you try the 349 mutant in this assay?

DR. WEISS: No, we did not do those experiments.

DR. GUISO: Do you have a similar expression of the pertussis toxin in your cyclase mutant and do you have a similar expression of cyclase as in the YA-5 in your pertussis mutant?

DR. WEISS: Yes, that is a good point actually. Adenylate cyclase mutant is dead minus. It makes zero activity and it makes zero antigenicity and it makes pertussis toxin. I am a geneticist, so I do like plus-minuses instead of micrograms or anything, so I know it makes pertussis toxin but I do not know how much and it would be important to look at that. The pertussis toxin mutant in contrast is deficient in activity. It is not dead negative, and it would be very nice to go back and do those experiments with a strain that made absolutely zero pertussis toxin activity. Maybe one of my colleagues who engineered those lovely mutants would give me one and I would be happy to try to it. It certainly makes lots of adenylate cyclase activity but at least I am convinced because the pertussis toxin mutant is so profoundly different in the lethal infection, that clearly it is very disabled in pertussis toxin production.

DR. KASLOW: Have you tried another mixing experiment which would be to infect with, say, the pertussis toxin minus mutant and then just inject pertussis toxin IV or IP and see whether systemic toxin allows that other bug to survive?

DR. WEISS: That is a good experiment. We have not done it.

DR. KASLOW: It might be useful because it would help identify the virulence target of pertussis toxin as to whether it is systemic or local which has implications for where you want the immune response to that antigen to occur as opposed to, say, the adhesins where it would be obvious I think that the mucosal immunity is important, but for pertussis toxin it is not so clear.

DR. WEISS: That is an excellent suggestion.

DR. PEPPLER: I was wondering if you looked in the lungs to see where the organisms are in those adenylate cyclase toxin minus mutants to see if they are inside cells, which cells were in the respiratory tract?

DR. WEISS: In these studies, no. We are starting to do some histologic studies and we have a lot to learn. They are very incomplete right now and I do not have any results to report.

DR. GOLDMAN: Given that all models of pertussis infection have their limitations, I was just curious as to what you think mouse lethality in the infant mouse system really reflects? I noticed that when you used lower doses in your sublethal challenges, you get a very long and high density infection, but they do not die. So why do you choose to use higher doses and measure mouse killing and then call that a reflection of this systemic disease pertussis?

DR. WEISS: Well, the reasoning we use the lethal infection is because it is easy, and I think it has given us a lot of information but I think we need to probably put that to rest and start doing more sophisticated models. Clearly adenylate cyclase and pertussis toxin are two important things to look at. Recovery from the lungs now, we see this phenomenon. We see a symmetry suggesting that adenylate cyclase works earlier and pertussis toxin works later. I think we needed to do very crude studies before we can be sophisticated about it, so I would love to do something a little more sophisticated in the future.

DR. SHAHIN: Can I just comment briefly on that point, Bill? These constructs are all Tohama I based, right?

DR. WEISS: Right.

DR. SHAHIN: With 18323, when you get above 10^7-10^8 in the mice, you generally see death. So that there may be some background differences also and I just think we need to, as Alison says, interpret our models with caution.

DR. WEISS: We can definitely kill them with the wild-type strain by giving them more but that does not tell you anything more.

DR. THOMAS: I was interested in your summary slide where you suggested that PT was immunosuppressive. Would you like to just briefly comment on the inference?

DR. WEISS: That was some of Bruce Meade's work. I guess immunosuppressive is probably not correct. He has shown that it interferes with chemotaxis and other things. I am sorry if I meant to allude something else.

DR. LEININGER: Have you looked at the colonization of the trachea?

DR. WEISS: No, we probably should but again those are very difficult experiments so we have done the easy things first, hoping no one would ask us to do the hard things.

Bordetella pertussis Adenylate Cyclase: A Protective Antigen against Lethality and Bacterial Colonization in Murine Respiratory and Intracerebral Models

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In developed countries where immunization levels with killed whole-cell *Bordetella pertussis* vaccine have remained high, children have been well protected against whooping cough (6). Because adverse side effects have been attributed to this whole-cell vaccine, however, some countries have stopped their immunization program and initiated studies on a new, safe acellular vaccine. After these periods of reduced immunization, the incidence of the disease always increases, indicating that (i) it is important to maintain high levels of immunization, (ii) protection with the current whole-cell vaccine is still incomplete, and (iii) *B. pertussis* is latent among healthy carriers.

What is needed is a safe vaccine with a better protective efficacy, i.e., one able to protect not only against the disease but also against the infection, with or without clinical expression. The major problem in formulating such a new acellular vaccine is to determine which antigen(s) to include. Two components of *B. pertussis*, pertussis toxin (PT) and filamentous hemagglutinin (FHA), have received considerable attention as potential vaccine candidates (1, 14, 16). Many studies have demonstrated that both active and passive immunizations against PT and FHA can protect mice against a respiratory challenge with *B. pertussis* (16). Other antigens may also prove to be effective vaccine components including agglutinogens such as the 69-kDa protein, other outer membrane proteins, and the bifunctional protein adenylate cyclase-hemolysin (AC-Hly).

The idea of using AC as a virulence determinant and protective antigen arose recently. Confer and Eaton (4) and Hanski (8) first showed that this protein inhibits macrophage and lymphocyte metabolism *in vitro*. Weiss et al. later generated a panel of mutants to show that those mutants deficient in AC-Hly production were severely impaired in their ability to cause a lethal infection in the infant mouse respiratory infection model (20, 21). Recently, Guiso et al. showed that passive and active immunizations with AC protect mice against pulmonary lesions induced by highly virulent variants of *B. pertussis* and *B. parapertussis*, thereby suggesting a role as a protective antigen for this molecule (7). Although Novotny et al. (11, 12) had already advanced this suggestion, it was later demonstrated that the protective activity observed in their study was due to a 69-kDa membrane protein, now characterized as an agglutinogen (2) contaminated by AC (3).

The present investigation examined the role of AC (i) as a protective antigen against lesions induced by *B. pertussis* in the adult mouse respiratory and intracerebral models and (ii) in the colonization of the host by the bacteria. The study showed that active immunization with AC can significantly reduce *B. pertussis* colonization in both the lungs and brains of mice. Thus, anti-AC immunity not only protects against cytotoxic effects but also against bacterial survival on target tissues.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study were lung-passaged derivatives of *B. pertussis* 18323 (type strain ATCC 9797), isolated as described previously (7). Cultures were done on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood (BG plate) at 36°C for 48 h. Subcultures in liquid media were performed on Stainer-Scholte medium (17) for 20 h at 36°C until adsorbance, measured at 650 nm, reached 1.0.

Adenylate cyclase. The enzyme was purified from culture supernatants using calmodulin affinity chromatography, as described previously (10). Our preparation consisted of two polypeptides of 45 and 43 kDa which are structurally related as described by Ladant et al. (10).

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* Presenting author.

Adenylate cyclase assay. AC activity was measured as described previously (10). One unit corresponds to one nmol of cAMP formed per min at 30°C at pH 8.

Vaccines. The lung-passaged derivatives were grown in Stainer-Scholte broth until they reached an absorbance of 1.0 at 650 nm; they were then heat-killed (30 min at 56°C). Whole-cell vaccine suspensions were diluted in saline to 5 x 10^8 CFU per dose and adsorbed on aluminum hydroxide (1 mg/ml) prior to use.

Active immunizations. For active immunizations, groups of 3-week-old BALB/c mice were given subcutaneously two injections of 5 µg purified AC adsorbed on aluminum hydroxide at a 2-week interval. Controls were injected with aluminum hydroxide alone. The mice were bled 12 days after the last injection to assess the presence of circulating anti-AC antibodies. Lethal or sublethal challenges were made 14 days after the final injection. Lethal challenges were made either by the intranasal (i.n.) administration of 2 x 10^7 CFU or intracerebral (i.c.) administration of 2 x 10^5 CFU. Sublethal challenges were performed either by i.n. administration of 7 x 10^5 CFU or i.c. injections of 10^3 CFU. Mice were observed for 14 days, with deaths recorded daily. Nonlethally infected mice were sacrificed by cervical dislocation 1 h after exposure (designated as day 0) and at various days thereafter. Six mice were sacrificed per time point. Each sacrificed mouse's lungs or brain were removed and homogenized in saline using tissue grinders. Dilutions of lung or brain homogenates were sampled on BG plates; CFU were counted after 3 to 4 days of incubation at 36°C.

Statistical analysis. Data were tested for statistical significance using student *t* test.

RESULTS

Protective efficacy of AC against lethality in murine i.n. and i.c. models. There are only two known organs in the mouse in which *B. pertussis* can multiply: the brain and the respiratory tract. Comparative studies of mouse protection potency by i.n. and i.c. methods showed that the i.c. model resulted in a much better correlation with whole cell vaccine efficacy in humans (6). Consequently, the i.c. model is still used as the reference method for potency testing of whole cell vaccine throughout the world. However, the i.n. model is more useful for studying the disease caused by *B. pertussis* and the protective efficacy of purified antigen since it is closer to the natural pertussis infectious process in several ways. This study thus compared both models to check the protective efficacy of purified AC.

In a previous study using the i.n. model, Guiso et al. reported that derivatives of *B. pertussis* from lung homogenates of mice infected by this organism were more virulent than the parent strain and could induce a lethal edematous hemorrhagic alveotis in adult mice (7). Hypothesizing that lung-passaged *B. pertussis* derivatives had enhanced virulence/antigenicity, we used them for infectious challenges of mice and to prepare whole-cell vaccines. AC used for active immunizations was purified to homogeneity from culture supernatants of lung-passaged *B. pertussis* derivatives. AC, which has a molecular weight of 45,000, is the fragment of the bifunctional AC-Hly protein carrying the AC activity stimulated by calmodulin (5); it is not, as shown by Rogel et al. (15), cytotoxic *in vitro*.

The efficacy of active immunizations with purified AC was evaluated by comparing them to whole cell vaccines in mice injected subcutaneously and subsequently le-

TABLE 1. Active protection against intranasal injection of *B. pertussis* with purified AC

Immunizing Antigen	Number of mice: Challenged	Survived	Survival %
AC	66	57	86
Vaccine	60	56	93
Controls	89	10	11

Mice were immunized two times with a 2-week interval with 5 µg of AC or 5 x 10^8 killed bacteria adsorbed to aluminum hydroxide. The lethal challenge of *B. pertussis* (2 x 10^5 CFU) was given 14 days after the last injection.

TABLE 2. Active protection against intracerebral injection of *B. pertussis* with purified AC

Immunizing Antigen	Number of mice: Challenged	Survived	Survival %
AC	10	8	80
Vaccine	10	9	90
Controls	21	1	5

Mice were immunized two times with a 2-week interval with 5 µg of AC or 5 x 10^8 killed bacteria adsorbed to aluminum hydroxide. The lethal challenge of *B. pertussis* (2 x 10^5 CFU) was given 14 days after the last injection.

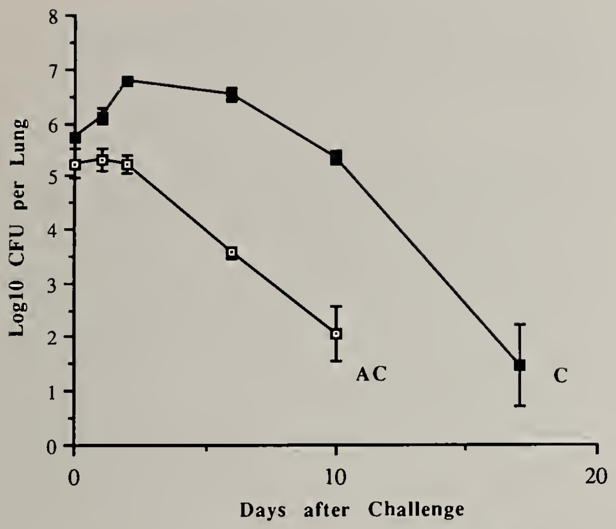

FIG. 1. *B. pertussis* 18323 colonization of the lungs of BALB/c mice actively immunized with purified adenylate cyclase. Mice were immunized two times with a 2-week interval with 5 µg of adenylate cyclase adsorbed to aluminum hydroxide. The sublethal challenge (7 x 10^5 CFU) was given intranasally 14 days after the last injection. Control mice (control) received aluminum hydroxide alone. The plots show the geometric mean standard deviation (bars) for six mice per time point.

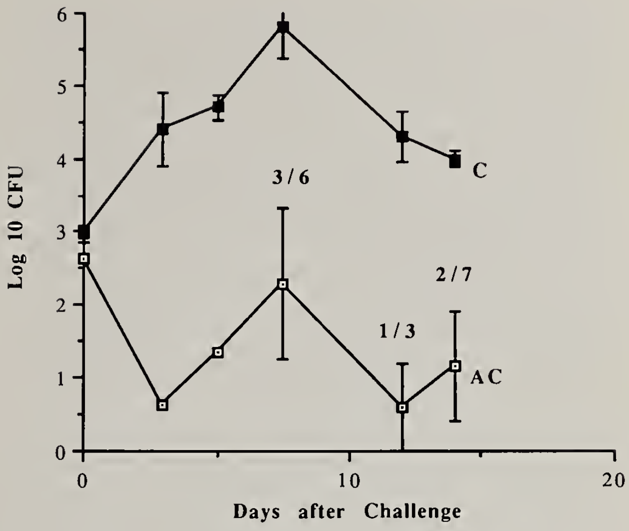

FIG. 2. *B. pertussis* 18323 colonization of the brains of BALB/c mice actively immunized with purified adenylate cyclase. Mice were immunized two times with a 2-week interval with 5 µg of adenylate cyclase adsorbed to aluminum hydroxide. The sublethal challenge (10^3 CFU) was given intracerebrally 14 days after the last injection. The numbers indicated on the curve are the number of mice colonized for the total number of mice.

thally challenged either by i.n. or i.c. injection with *B. pertussis* lung-passaged derivatives. As shown in Tables 1 and 2, immunization with purified AC was as protective as whole cell vaccine in both respiratory and intracerebral challenges.

Protective efficacy of AC against bacterial colonization in murine i.n. and i.c. models. The role of AC as a protective antigen against colonization of the mouse respiratory tract was evaluated. In control mice receiving only aluminium hydroxide, bacterial counts in the lungs increased from 5 x 10^5 CFU immediately after i.n. challenge to 6 x 10^6 CFU at days 2 and 3 after challenge (Fig. 1). Immunization with AC does not allow the bacteria to multiply and differences of 1.5 and 3 logs were observed between control and immunized animals at days 2 and 8, respectively.

The role of AC was also evaluated as a protective antigen against bacterial colonization of the mouse brain. The number of CFU in the brains of mice immunized with AC was considerably reduced compared with the number for control mice and never increased above the initial challenge level (Fig. 2). Furthermore, at day 8, half of the immunized mice had already cleared the bacteria and were fully protected; only 2 out of 7, compared to 6 out of 6 in the control group, remained infected at day 14.

DISCUSSION

A pertussis acellular vaccine should be devoid of toxic components but have a better protective efficacy than the current inactivated whole-cell vaccine. It should not only protect individuals against disease but also reduce asymptomatic carriage of *B. pertussis*. The inclusion of a particular antigen in an acellular pertussis vaccine will depend on its role in pathogenesis and demonstration of its protective potency in a relevant animal model. Thus, the vaccine should include both adhesins and toxoids.

FHA and PT are presently the main vaccine candidates. FHA has been chosen by several groups because it is devoid of toxic properties and because of its prominent role in adhesion of *B. pertussis* to the ciliated cells of the host respiratory tract (18). PT was chosen because of its important toxic role during pertussis disease (19). However, PT does not seem to be the only toxin since *Bordetella parapertussis* and *Bordetella bronchiseptica* elicit pertussis-like disease in humans and mice, but fail to express functional PT.

Among those factors expressed by all three *Bordetella* species which could contribute to the disease, the AC-

Hly molecule is a good candidate since (i) mutants deficient in the expression of this protein are severely diminished in their ability to cause lethal infection in mice, (ii) purified AC-Hly can penetrate mammalian cells *in vitro* and disturb cellular functions, and (iii) highly virulent derivatives of *B. pertussis* and *B. parapertussis* express higher levels of AC-Hly than do parental strains (7, 8, 20, 21). Consequently, this study examined the role of AC-Hly as a protective antigen. We used the AC fragment of the AC-Hly because of its better recovery during the purification process and because this form is devoid of toxicity *in vitro* (15). To test the protective efficacy of this antigen, we compared two animal models: respiratory and intracerebral infection. The study included both models to check AC's protective efficacy because (i) the respiratory infection closely mimics natural pertussis and (ii) the intracerebral model is used in many studies on the protective efficacy of vaccines or purified antigens.

The findings presented here demonstrate that AC is a protective antigen against the disease induced in mice by *B. pertussis*. Using either respiratory or intracerebral challenges, its efficacy of protection is similar to that of the whole-cell vaccine. As PT, the AC antigen has similar protective efficacy in both animal models, again suggesting an important role in the pathogenesis of *B. pertussis*. Moreover, as shown in Figs. 1 and 2, the AC fragment is also a protective antigen against bacterial colonization and multiplication. Immunization with AC thus protects mice 1 day after infection since there is no multiplication of bacteria; the number of bacteria actually decreased at day 2. Both respiratory and intracerebral infection models display similar features. In comparison, bacterial growth occurs in the control mice.

The *in vitro* studies suggested that AC-Hly may help *B. pertussis* survive and better multiply on the surface of the respiratory epithelium by locally depressing the immune system (8). This hypothesis could explain how immunization with AC protects mice against bacterial colonization since the AC-Hly molecule has no demonstrated role in bacterial adhesion (21). The fact that protection against bacterial colonization by AC occurs very early after infection suggests that AC-Hly is acting immediately after adhesion of the bacteria on the target cells. This supports the hypothesis that HC-Hly is delivered by direct interaction of the bacterium with target cells and is acting locally (9). More work is needed to determine the AC-Hly target cell and to fully understand its role in the virulence and survival of *B. pertussis*, in vivo.

ACKNOWLEDGMENTS

This work was supported by funds from the Pasteur Institute Foundation and Pasteur Vaccins.

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DISCUSSION

DR. LEININGER: Are you saying that adenylate cyclase is functioning as an adhesin?

DR. GUISO: I have absolutely no proof of that. Maybe, but I do not think so. I think that the major function is probably the immunosuppressive function as Alison Weiss told you before but I have absolutely no argument for that.

DR. RAPPUOLI: If I remember correctly, a few years ago I saw a paper, which suggested that the *Bordetella pertussis* adenylate cyclase is cross-reacting with the human brain adenylate cyclase. Is that true? In the case that that is true, will you propose that as a vaccine?

DR. GUISO: Agnes Ullmann showed a cross-reaction with brain adenylate cyclase, that is true. I did not find any. For me, there is no cross-reaction. That is only thing that I can answer.

DR. ULLMANN: It has been shown that there is a cross-reaction due to a peptide which is the ADP binding site which is common for *Bordetella pertussis, Bacillus anthracis* adenylate cyclase. Antibodies against these peptides are recognized perfectly well brain adenylate cyclase. So if the peptide is inside the brain adenylate cyclase molecule then you cannot see it but definitely it represents at least a seventeen amino acid peptide in the substrate binding region.

DR. GUISO: Yes, I just want to add also that Agnes is entirely right about that. We did the same experiment using the *Bacillus anthracis* adenylate cyclase and we had absolutely no protection at all. Then it is an entirely different molecule concerning the protective activity.

DR. HEWLETT: I wondered if you looked at the serum from these mice that have been protected afterwards to see whether they have antibody against the cyclase, whether it neutralizes the enzyme or toxin activity and whether there is antibody there against any other products. For example, when we purified adenylate cyclase and it is one band on a gel, there is sometimes a little bit of either FHA or PT or 69 kDa that contaminates the preparation which we can only see by Western blot.

DR. GUISO: I can answer you that all groups of mice immunized with the adenylate cyclase fragment possess a high titer of antibodies against the 45 but also against the 200 kDa protein, that those serums do not recognize the FHA or the PT protein, and that sometimes the serum inhibits the enzyme activity but not all. That is it. As you observed in human, we observed after an infection in mice as during a pertussis disease in infant because we have as you some preliminary results in our laboratory that antibodies against adenylate cyclase appear very early after the infection, especially in newborns. Then it is a very early marker.

SESSION FOUR

Molecular Genetics and Regulation of Virulence Factors

Co-chairpersons:

Camille Locht
E. Scott Stibitz

Introduction

E. SCOTT STIBITZ

I would like to welcome you all to the session on Molecular Genetics and Regulation of Virulence Factors and to just say a few words in outset. I think the power of the molecular approach to the study of *Bordetella pertussis* has already been well documented in this meeting. It is quite remarkable to think about the contributions that have been made in a really relatively short period of time. Although I do not think of myself as an old timer, I find I am able to recall the good old days of molecular genetics and pertussis.

It is safe to say, I do not think many would argue with the statement, that this field really got off the ground with the work of Alison Weiss when she developed a way to do transposon mutagenesis in *B. pertussis* and as a result of this, she was able to define by mutation the genetic loci for pertussis toxin, adenylate cyclase hemolysin, FHA and vir. The mutants she made have been used in numerous studies and have been sent all around the world and I know this for a fact because I used to have to send them out personally. But as an illustration of the impact of these initial studies had, I remember two years ago at the meeting in Hamilton, Montana, John Gotto was heard to say something to the effect of, well, my presentation is unique because I did not used the Tn*5*s of Alison Weiss and he went on to add and I am not collaborating with Rino Rappuoli.

Now, Alison was a graduate student in Stan Falkow's laboratory when she did this work, and I would just like to remind everyone that we are also indebted to Margaret Pittman because, as the story goes, she turned to Stanley at a banquet one time and said something to the effect of, if you are so smart, why don't you work on pertussis. And the rest is history.

Regulation of *fhaB, bvg,* and *ptx* Transcription in *Escherichia coli:* a Comparative Analysis

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The *Bordetella* virulence regulon comprises a large array of loci that are activated or repressed, directly or indirectly, by the *bvgAS* (*vir*) sensory transduction system (1, 12, 31, 33). Transcription of the pertussis toxin operon (*ptx*), the cyclolysin gene (*cyaA*), the locus encoding filamentous hemagglutinin (*fhaB*), and the *bvg* operon itself requires the *bvg* products (6, 15, 17, 25). In addition, expression of dermonecrotic toxin, fimbriae, a 69-kDa outer membrane protein, cytochrome d-629, and several loci identified by genomic fusions are positively controlled by *bvg* (12, 17, 31). Negative regulation has also been described, and at least five loci are controlled in this manner (12).

Two related forms of coordinate regulation control the expression of virulence in *Bordetella pertussis.* Phase variation is a reversible metastable transition from virulent to avirulent phases that results from an alteration at the *bvg* locus (29). The second type of regulation involves a sensory transduction system that controls expression of the virulence regulon in response to environmental signals (14). Growth in the presence of $MgSO_4$, nicotinic acid, low temperature (25°C versus 37°C), and other conditions decreases expression of *bvg*-activated genes and derepresses expression of *bvg*-repressed genes. The role of this sensory system in pathogenesis is unknown.

The nucleotide sequence of the bvg region includes two open reading frames, *bvgA* and *bvgS* (1; S. Stibitz, personal communication). The *bvgA* encodes a 24-kDa polypeptide (26), whereas the *bvgS* produces a 134-kDa protein (S. Stibitz, personal communication). In the initial DNA sequence, a single nucleotide error resulted in the assignment of three open reading frames to the *bvg* locus (1). The predicted amino acid sequences of *bvgA* and *bvgS* share extensive homology with a family of bacterial proteins that respond to environmental signals (22, 24). These systems usually consist of at least two regulatory proteins, one of which contains a C-terminal "transmitter" domain (ca. 250 amino acids), and the other of which carries an N-terminal "receiver" domain (ca. 115 amino acids) (13). Biochemical evidence from studies of nitrogen regulation (11), chemotaxis (8), and osmo-regulation (10) indicates that transmitter proteins are kinases that respond to environmental stimuli and modulate the activity of receiver proteins through phosphorylation. Receiver proteins are usually involved in transcriptional regulation, although other effector functions have been described (8). The N-terminus of *bvgA* contains a receiver, and the predicted sequence of *bvgS* contains a transmitter. The *bvgS* sequence is unusual, however, in that it also contains a receiver domain located near its C terminus (1).

The complexity of the *bvg* regulon is evident from the large number of regulated loci. Although the *bvgAS* products appear to play a central role in sensory transduction and subsequent control, the regulatory system is likely to reflect the complexity of the regulated factors. A system that uses transcriptional fusions in *Escherichia coli* to analyze *cis*- and *trans*-acting components from the virulence regulon of *B. pertussis* has been described, and the *bvg* locus was shown to be sufficient for both transcriptional activation and sensory transduction (19, 25, 26). This report presents data relating to regulation of *fhaB*, *ptx*, and *bvg*, and extends these findings to support the hypothesis that differential requirements exist for the regulation of these *bvg*-activated loci.

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* Presenting author.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The *E. coli* strains and plasmids used in this study are described in Table 1. Bacterial strains were grown in L-broth or on L-agar plates (16) unless otherwise indicated. Antibiotics were added to liquid or solid media at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 35 μg/ml; and chloramphenicol, 30 μg/ml. X-gal (5-bromo-4-chloro-3-indoyl-*B*-D-galactoside) was dissolved in dimethylformamide and added to media at a final concentration of 40 μg/ml.

Recombinant DNA methods. Standard methods were used for the isolation of plasmid DNA, restriction enzyme digestions, calf intestinal phosphatase treatment, conversion of 5′ overhangs to blunt ends with Klenow fragment, agarose gel electrophoresis, DNA ligations, and transformation of plasmid DNA (16). High-efficiency DNA transformation was accomplished by electroporation of electrocompetent cells using a gene pulser (Bio-Rad Laboratories, Richmond, Calif.) as previously described (5). DNA fragments were isolated from low melting point agarose (Sea Plaque, FMC Corp., Marine Colloids Div., Rockland, Maine) by extraction with buffer-saturated phenol followed by chloroform extraction and ethanol precipitation prior to ligation.

Construction of *lacZYA* transcriptional fusions. The DNA restriction fragments (Fig. 2) were isolated from plasmid pUW1004 (30) and inserted into pRS551 (28). This latter plasmid contains a short polylinker followed by a distal portion of the *trpA* gene, the *trpT* sequence, the W205 *trp-lac* fusion junction, and the intact *lacZ* gene including its wild-type translation initiation site but missing transcriptional control sequences. The fusion sites are preceded by four tandem copies ($T1_4$) of the strong transcriptional terminator from the *E. coli rrnB* ribosomal RNA operon, the *kan* gene from Tn903, and the *bla* gene from pBR322.

Fusions carried by pRS551 derivatives were transferred onto λRS45 by in vivo homologous recombination. λRS45 is a bacteriophage λ derivative which carries a nonfunctional 3′ portion of the *bla* gene from pBR322 preceding a DNA fragment which encodes the promoter distal third segment of *lacZ* followed by *lacYA*. Construction of λJMC3, λJME3, and λCR430 was previously described (19, 25).

β-galactosidase assays. MC4101 lysogens containing *lacZYA* transcriptional fusions were grown overnight at 37°C in L-broth or L-broth containing 40 mM of $MgSO_4$. Overnight cultures were diluted 1:100 in the same media and grown to an optical density (OD_{600}) of approximately 0.5. β-galactosidase activities were determined with cells permeabilized with SDS/$CHCl_3$ as described by Miller (18).

Construction of a *B. pertussis* cosmid library. A genomic library of *B. pertussis* BP536 (32) sequences was constructed by partial digestion of chromosomal DNA with Sau3A to produce fragments averaging 35 to 40 kilobases (Kb). These fragments were mixed with the *Bam*HI-digested, calf intestine alkaline phosphatase-treated cosmid vector pHC79 (9) and ligated. Ligation products were packaged into bacteriophage capsids using GIGAPAK packaging mix (Stratagene Inc.).

RESULTS

bvg* regulation of transcriptional fusions in *E. coli. To study *trans*-acting factors required for transcriptional activation, restriction fragments containing the 5′ promoter regions of the *B. pertussis fhaB*, *bvg*, and *ptx* loci were inserted upstream of a promoterless *lacZYA* operon carried by the pBR322 derivative pRS551 (Table 1, Fig. 1) (19, 25, 26, 28). The resulting gene fusions were transferred from multicopy plasmids onto a bacteriophage lambda vector by in vivo homologous recombination, and *E. coli* MC4101 lysogens were constructed to allow analysis of single-copy transcriptional fusions.

The 2.84-kb *Eco*RI-*Bam*HI fragment present in λJMC3 encodes the N terminus of the *fhaB* structural gene and 253 base pairs (bp) of the 5′ noncoding region (4, 19). λJME3 carries a 931-bp fragment that includes the *ptx* operon promoter, the beginning of *ptxA*, and 481 bp upstream from the site of transcription initiation (21). The *ptx* sequences required for *bvg*-activated transcription in *B. pertussis* lie within a 170-bp region located 5′ to the mRNA start site (7). The λCR430 *bvgA::lacZYA* fusion contains both the *bvg*P1 and *bvg*P2 promoters, with +1 sites located 90 and 39 bp from the *Eco*RI recognition sequence, respectively (25).

The basal level of β-galactosidase activity expressed by λCR430 is five- to eightfold higher than the basal level observed for the other fusions (Fig. 1). This disparity is most likely due to the constitutive activity of the *bvg*P2 promoter (25). Addition of the *bvgAS* operon carried on plasmid pJM26 resulted in a 500-fold induction of *fhaB::lacZYA* transcription, a ninefold increase in *bvgA::lacZYA* expression, and no change in the level of *ptx::lacZYA* expression (19, 25). In addition, *bvg*-mediated activation responds to modulating agents such as $MgSO_4$.

The 432-bp region between the *fhaB* and *bvgA* structural genes contains divergent promoters which are *bvg*

TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain, plasmid, or bacteriophage	Description	Reference or source
***E. coli* strains**		
MC4100	F- araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR	27
MC4101	MC4100 recA1	19
DH5α	F- φ80d*lacZ* ΔM15 Δ(*lac*ZYA-argF)U169 *endA1 recA1 hsdR17* (rk- mk-) *deoR thi-1 supE44 gyrA96, relA1*	BRL
JFMC3	MC4101 (λJMC3)	19
JFME3	MC4101 (λJME3)	19
JFMC35	DH5α (λJMC3)	This study
JFME35	DH5α (λJME3)	This study
Plasmids		
pRS551	*lacZYA* transcriptional fusion vector derived from pBR322, *Eco*RI-*Sma*I-*Bam*HI polylinker, Ap^r Km^r	28
pJMC3	pRS551 with 2.84-kb *Bam*HI-*Eco*RI fragment from pUW1004	19
pJME3	pRS551 with 0.9-kb *Eco*RI-*Sal*I fragment from pTox27	19
pCR430	pRS551 with 0.53-kb *Eco*RI-*StyI* fragment containing *bvgA'*	25
pACYC18	cloning vector derived from p15A, compatible with *Col*EI-derived 4 replicons, Cm^r Tc^r	3
pUW1004	pACYC184 with 14.7-kb insert from *B. pertussis* encoding *bvgAS*, Cm^r	30
pBR322	Ap^r Tc^r cloning vector	2
pTox27	pBR322 with 0.9-kb *Eco*RI-*Sal*I insert from ptA region	W. J. Black
pJM26	pBR322 with 5.072-kb *Eco*RI-*Nde*I insert containing *bvgAS*	19
pKS-	High copy number cloning vector with polylinker, Ap^r	Stratagene
pCR301	pKS- with 3.1-kb *Bam*HI insert from pUW1004	This study
pCR414	pKS- with 1.1-kb PstI insert	26
pDR3.0	pKS- with 2.84-kb *Bam*HI-*Eco*RI insert	D. A. Relman
pCR412	pKS- with 1.244-kb *PstI* insert	This study
pDR7	pKS- with 0.502-kb *PstI*-*Eco*RI insert	D. A. Relman
pCR416	pKS- with 0.746-kb *Eco*RI-*PstI* insert	26
pCR428	pKS- with 0.53-kb *Eco*RI-*StyI* insert	This study
pCR450	pKS- with 0.13-kb *Eco*RI-*MluI* insert	This study
pJME30	pKS- with 0.931-kb *Eco*RI-*SalI* insert from pTox27	W. J. Black
Bacteriophages		
λRS45	λ*lacZYA* fusion vector for recombination with pRS plasmids containing transcriptional fusions '*bla-'lacZYA, imm21, ind+*, Ap^s Km^s	28
λJMC3	λRS45 x pJMC3 recombinant, Km^r	19
λJME3	λRS45 x pJME3 recombinant, Km^r	19
λCR430	λRS45 x pCR430 recombinant, Km^r	25

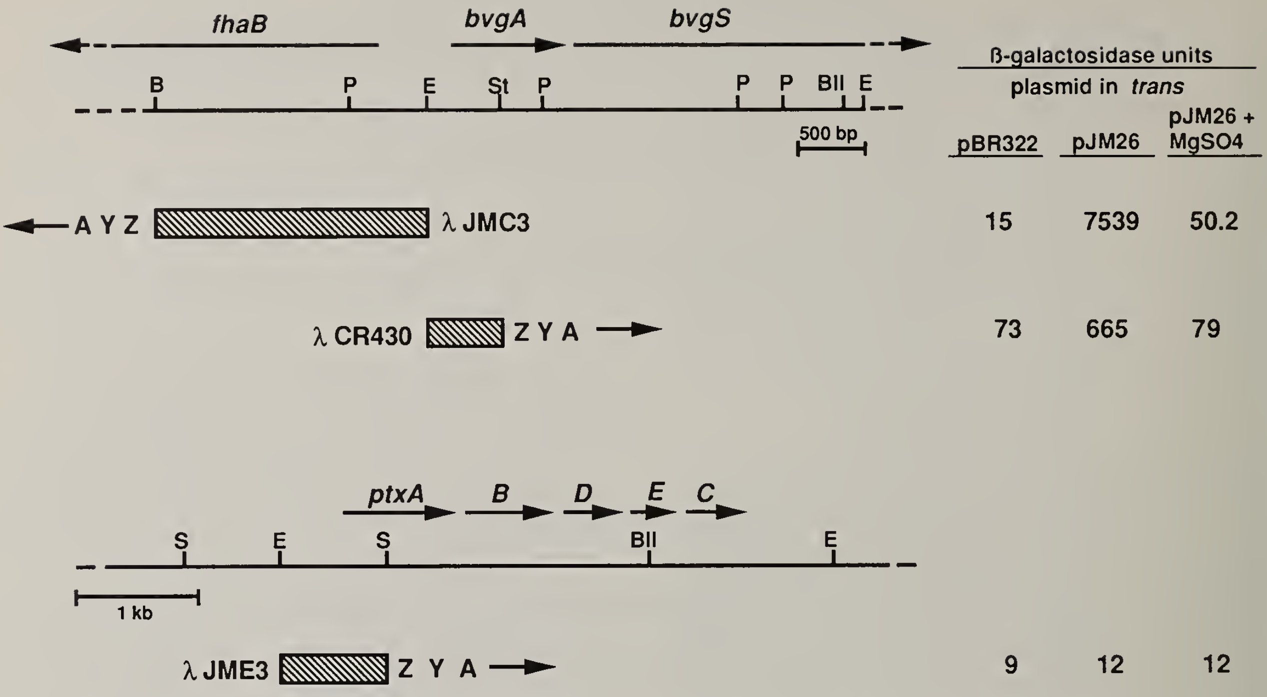

FIG. 1. Structure of bacteriophage λ derivatives carrying *lacZYA* transcriptional fusions. Partial restriction maps of the regions from the *B. pertussis* chromosome containing the *fhaB*, *bvg*, and *ptx* loci are shown (1, 20, 23, 30). Plasmid pJM26 is a pBR322 derivative carrying the intact *bvgAS* operon (Table 1). Bacteriophage λ derivatives carry the indicated fragments fused to *lacZYA* as described in text. Abbreviations for restriction sites: B, *Bam*HI; E, *Eco*RI; BII, *Bgl*II; N, *Nde*I; X, *Xho*I; S, *Sal*I; St, *Sty*I. β-galactosidase activities are average values obtained from at least three independent assays which differed by no more than 20%. Activities were determined as described in text and are reported as Miller units (18).

activated. Extension of the λJMC3 fusion to the *Bgl*II site in *bvgS*, and extension of the λCR430 fusion to the *Pst*I site in *fhaB*, has little effect on the basal or activated levels of β-galactosidase measured in *E. coli* (data not shown). This finding was somewhat surprising considering the possibility for regulatory interactions within this intergenic region, and it will be interesting to compare these results with analogous studies in *B. pertussis*.

Primer extension was used to map an *fhaB*::*lacZYA* transcription initiation site in strain JFMC3 carrying pJM26. This start site corresponds to a cytosine residue located 69 nucleotides upstream from the *fhaB* initiation codon. The observed transcript is *bvg* dependent and is not present in JFMC3(pJM26) grown in 40 mM of $MgSO_4$. These results show that initiation of *fhaB* transcription in *E. coli* occurs in the same region as previously identified in *B. pertussis* (4).

Effect on *trans* activation of multicopy plasmids carrying sequences from the 5′ regions of *fhaB*, *bvg*, and *ptx*. The *bvgA* product has been shown to function as a transcriptional activator (26). A possible explanation for the ability of plasmids carrying the *bvg* locus to *trans* activate *fhaB*::*lacZYA* expression to high levels is that *BvgA* acts at or near the *fhaB* promoter (19, 25). High copy number plasmids carrying *cis*-acting control sequences would be expected to titrate *trans*-acting regulatory factors that are present in limited supply. This prediction was tested using a single-copy *fhaB*::*lacZYA* fusion in *E. coli*, with the *bvg* operon carried by the intermediate copy number vector pACYC184.

High copy number *Col*EI derivatives carrying portions of the *fhaB*, *bvgA*, or *ptxA* loci were transformed into JFMC3 containing plasmid pUW1004. The pUW1004 normally activates JFMC3 *fhaB*::*lacZYA* expression by approximately 60-fold, giving induced activities averaging 790 units. The pCR301 and pCR414 had no effect on activation when compared to the vector pKS^- control (Fig. 2). JFMC3(pUW1004) carrying either of these plasmids expressed approximately 800 units of β-galactosidase and produced dark blue colonies on X-gal plates. The pCR301 and pCR414 contain internal restriction fragments from the *fhaB* structural gene and the *bvgAS* locus, respectively. In contrast, pDR3.0, which carries the *Eco*RI-*Bam*HI fragment also present in

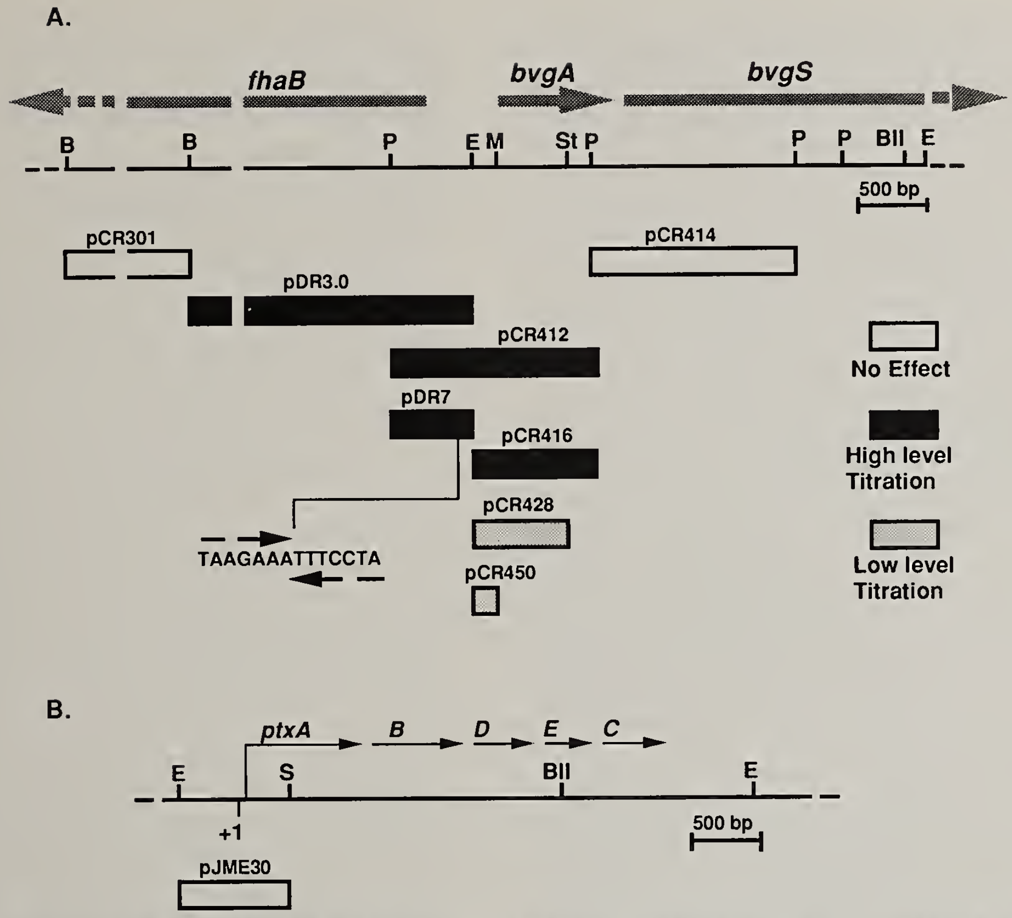

FIG. 2. Restriction fragments from the (A) *fhaB* and *bvgAS* or (B) *ptxA* regions of *B. pertussis* carried on high copy number *ColE*1 derivatives (Table 1). Open rectangles indicate that the plasmids had no effect, as compared to the vector pKS$^-$ control, on *fhaB*::*lacZ* expression in JFMC 3 (pUW1004).
■ indicate plasmids that decreased *fhaB*::*lacZ* expression in JFMC3 (pUW1004) to basal levels.
▨ indicate sequences that have a moderate effect (approximately 25% decrease) in the assay. Details of the assay are presented in the text. The 14-bp inverted repeat present upstream of the *fhaB* locus is also shown. Abbreviations for restriction sites are the same as in Fig. 1.

λJMC3, resulted in a marked reduction in *fhaB-lacZ* expression. JFMC3(pUW1004, pDR3.0) produced approximately 50 units of β-galactosidase and pale blue colonies on X-gal plates. The pJME30, which contains the *ptx* operon promoter and upstream regulatory region carried by λJME3, was completely inactive in the titration assay.

The pCR412 and pDR7 contain the 502-bp *Eco*RI-PstI fragment which includes the N terminus of the *fhaB* structural gene and 250 bp of upstream sequences. These plasmids had the same effect in JFMC3(pUW1004) as did pDR3.0, suggesting that the 502-bp fragment is sufficient for the observed decrease in *trans* activation. Recent analysis in *E. coli* indicates that a 14-bp palindromic sequence located 97 bp upstream from the *fhaB* +1 site is required for *trans* activation by *bvg* (C. Roy and S. Falkow, manuscript in preparation). Insertion of an oligonucleotide encoding this sequence into the pKS$^-$ vector results in a plasmid with titrating activity indistinguishable from pDR7 or pDR3.0 (Fig. 2).

The 742-bp *Eco*RI-*Pst*I fragment carried by pCR416 also efficiently decreased *bvg*-dependent expression of *fhaB*::*lacZ*. This fragment contains the first 561 bp of the *bvgA* structural gene and 181 bp of upstream sequences. Since pCR416 encodes most of the *bvgA* gene product, the effect of plasmid pCR428, which produces only a small portion of this protein, was next examined. The pCR428 reduced β-galactosidase expression when placed in *trans* to JFMC3(pUW1004) by approximately 25% in the assay. A similar reduction in β-galactosidase activity was observed with pCR450, which contains the *bvgA* promoters but none of the structural gene. The truncated BvgA protein expressed by pCR416 is apparently required for high-level inhibition of *trans* activation of the reporter fusion in JFMC3 by plasmids containing only *bvg* sequences.

These results suggest that there are several pathways by which high copy number plasmids carrying sequences from *bvg*-regulated genes can interfere with transcriptional activation of *fhaB*::*lacZYA* expression (see below). Furthermore, the *bvg*-activation ratio observed for a given gene fusion in *E. coli* appears to correlate with the effect of its promoter region in the titration assay.

Effect of cosmid clones from a *B. pertussis* gene bank on *bvg*-mediated activation of *ptx* transcription. The results described above are consistent with the hypothesis that *bvg* directly activates *fhaB* and *bvg* transcription, whereas one or more additional regulatory factors are needed for expression of *ptx*. In an initial attempt to identify such a factor, a gene library containing *B. pertussis* sequences in the cosmid vector pHC79 was constructed (9). Restriction analysis of several independent clones showed random cleavage patterns and inserts of approximately 40 Kb.

The r^+m^+ phenotypes of MC4101 derivatives resulted in a 1,200-fold decrease in the efficiency of cosmid plating as compared with DH5α (r^-m^+). For this reason, DH5α lysogens were constructed with λJME3 (*ptx*::*lacZYA*) and λJMC3 (*fhaB*::*lacZYA*) serving as controls. The resulting strains, JFME35 and JFMC35 as well as JFME35 carrying pUW1004, were infected with the cosmid library and plated on selective media containing X-gal. Out of the approximately 6,000 JFMC35 transfectant colonies examined, 8 were Xg-blue. Upon further analysis these clones were found to carry different cosmids sharing restriction digestion fragments identical to those at the *bvg* locus. It was concluded from this control experiment that the library carried the *bvg* locus at a frequency of about 1/750 cosmids. In contrast, infection of JFME35 or JFME35(pUW1004) with the cosmid library did not give rise to Xg-blue colonies, despite the screening of nearly 6,000 JFME35 and over 10,000 JFME35 (pUW1004) transfectants. These results suggest that no single member of the cosmid library is sufficient to allow *bvg* activation of *ptx* expression in *E. coli*.

DISCUSSION

The *bvg* products are sufficient for *trans* activation of *fhaB* and *bvgA* transcription in *E. coli* (19, 25). Furthermore, the level of activation responds to the same environmental signals as observed in *B. pertussis*. Several *B. pertussis* sensory transduction mutants have recently been characterized. The mutations resulting in constitutive activation map to the *bvgS* locus and retain their phenotype in the *E. coli* system (J. F. Miller, N. Black, and S. Falkow, manuscript in preparation). It is therefore concluded that the effects of the *bvg* locus on *fhaB* and *bvgA* transcription in *E. coli* reflect its activity in *B. pertussis*. Accordingly, this study has exploited the approach of analyzing *cis*- and *trans*-acting regulatory components in *E. coli*, followed by comparative experiments in *Bordetella* (19, 25, 26).

The ability of high copy number plasmids carrying 5′ regions of *fhaB* to decrease expression of *fhaB*::*lacZ* fusions is consistent with a model in which *cis*-acting regulatory sequences titrate *trans*-acting factors. Titration correlates with the presence of a 14-bp indirect repeat found upstream of *fhaB* which is sufficient for full activity. Deletion analysis and in vitro binding studies have recently shown that BvgA binds to this sequence to activate transcription at the *fhaB* promoter (Roy and Falkow).

The effect of plasmid pCR416 on *bvg* activation of *fhaB*::*lacZYA* is similar to that of the *fhaB* inverted repeat, although the mechanism appears to be quite different. The pCR416 encodes a nearly full size BvgA protein (Fig. 2). Deletions that decrease or entirely remove *bvgA* structural gene sequences, yet retain the *bvg* promoter, reduce the high level of titration observed for this plasmid. The pCR416 produces a truncated BvgA protein that is inactive in transcriptional control (26) but able to inhibit *trans* activation by *bvg*. There are several mechanisms that might account for this finding, including the formation of inactive multimers or perhaps the ability of truncated BvgA proteins to compete with the wild-type product for phosphorylation by *bvgS*. Experiments addressing these possibilities are currently in progress.

The promoter and regulatory region of the *bvg* operon is not as active in the titration assay as plasmids containing *fhaB* upstream sequences, although the *bvg* upstream region responds to the presence of *bvg* products as shown by induction of the *bvgA*::*lacZYA* fusion. The disparate effects of high copy number *fhaB* and *bvg* promoter regions on *trans* activation correspond to the induction ratios observed in *E. coli* (500-fold versus 9-fold, respectively). The ability of a restriction fragment at high copy to inhibit *trans* activation probably depends on several factors which include the copy numbers of *cis*- and *trans*-acting regulatory elements, as well as the association constants characterizing the relevant DNA-protein interactions.

In *B. pertussis*, an intact *bvg* locus is required for transcription of the *ptx* operon as measured by primer extension, mRNA hybridization, and transcriptional fusions to the chloramphenicol acetyltransferase gene (7, 17, 21, 26). In contrast, this study's results demonstrate that *bvg* is not sufficient for transcription of a *ptxA::lacZYA* fusion in *E. coli*. Additionally, the *ptx* promoter and upstream regulatory region have no effect in the titration assay described above. These observations suggest that one or more factors required for expression of *ptx*, but not *fhaB*, are absent or inactive in the reconstituted system. It is therefore possible that additional regulatory factors exist, and that the *Bordetella* virulence regulon is organized in a hierarchical fashion. Efforts to identify single cosmids able to promote *bvg* activation of *ptx* transcription in *E. coli* were unsuccessful, indicating that several unlinked regulatory factors are needed or that the *E. coli* transcriptional apparatus is unable to interact productively with additional elements required at the *ptx* promoter.

It is interesting to speculate on the relative placement of factors in the virulence regulon. To date, only two *bvg*-regulated loci have been shown to respond to the *bvgAS* products directly in *E. coli*. These loci encode the *bvg* sensory transduction system as well as FHA, an important attachment factor. It has been proposed that *bvg* and *fhaB* represent the top level of a hierarchical regulatory system (25, 26). Upon transfer from a modulated environment, activated BvgA would directly promote its own expression as well as that of *fhaB*. The result would be amplification of the regulatory system as well as production of the FHA adhesin. Efficient attachment to respiratory tissue would then allow the infectious cycle to proceed.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI23945 from the National Institutes of Health. J.F.M. was supported by Damon Runyon-Walter Winchell postdoctoral fellowship DRG-911.

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DISCUSSION

DR. WEISS: Your source for the bvgA in *E. coli*. Do you think that, can you hypothesize about that, do you have any evidence . . . (inaudible).

DR. ROY: The in vivo titration data which was given in the manuscript would presumably be measuring the phosphorylated bvgA in vivo and with that data, we do not have evidence for binding because it cannot titrate.

DR. WEISS: Is this *E. coli* in vivo?

DR. ROY: This is *E. coli* in vivo. But every line of evidence suggests it is phosphorylated in *E. coli* with the other factors, with the other *bvg* genes present. Otherwise, modulation would not occur in *E. coli*. As far as the in vitro data go, these two component regulatory systems, the transcriptional activators usually bind in the non-phosphorylated state. There is a question of affinity which has not really been addressed in any of the systems and is a difficult question to address, and it is reasonable to assume that the affinity is different, however, so different that you would not get binding in vitro. It has not been shown that it is that different.

DR. RAPPUOLI: Your model is mutually exclusive. As you know, we are finding some . . . (inaudible).

DR. ROY: Mutually exclusive means in a given cell they are mutually exclusive. In a population presumably you could get expression of both of them. However, the protein binding would suggest, in a given cell you would either be expressing one or the other but as activator falls on and off, these could actually be oscillating in a population as well as with that phase variation in culture results in a higher level of expression of P2 so in a population sense, it is not unusual probably to see both, and in fact, when we look at *E. coli*, we see both presumably because it is multi-copy and you just cannot shut all of them down.

DR. ULLMANN: Do you have full length message for ADP or do you have some (inaudible) examination, enough to use?

DR. ROY: Based on fusions, the closer you make the fusion, fusions and *bvgA* show a higher amount of activity than fusions in *bvgC*. Now, whether that is through actual terminators in the operon or just transcriptional polarity on the operon we do not really know but I think Rino knows this, too, through Western blotting that if you look at products, the *bvgA* gene product appears to be amplified much more than the other products.

Genetic Studies on the *vir* Locus of *Bordetella pertussis*

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Bordetella pertussis, the causative agent of whooping cough, synthesizes a number of molecules believed to be involved in the ability of this organism to colonize the human host and evoke subsequent pathology. Factors for which genetic, cytochemical, or immunological evidence supports such a role are pertussis toxin (PT), filamentous hemagglutinin (FHA), adenylate cyclase toxin/hemolysin (AC/Hly), a 69-kDa outer membrane protein now termed pertactin (Ptn), and tracheal cytotoxin.

With the exception of tracheal cytotoxin which is apparently a byproduct of peptidoglycan synthesis, the synthesis of the above virulence determinants has been found to be under the control of a separate genetic locus called *vir*. The *vir* locus was identified by Weiss et al. when it was shown that single insertions of the transposon Tn*5* within this locus simultaneously abolished the synthesis of PT, FHA, AC/Hly and Dnt (16). It has since been shown that pertactin is also regulated by the *vir* locus (4). The synthesis of *vir*-regulated factors is affected by environmental conditions, a phenomenon known as antigenic or phenotypic modulation (6, 7). Under certain culture conditions such as in the presence of $MgSO_4$ or nicotinic acid, or at reduced temperatures, the synthesis of *vir*-regulated factors is repressed. The *vir* locus is believed to mediate this environmental responsiveness.

The *vir* locus has also been shown to be the site of mutations giving rise to a phenomenon which has been called phase variation. This term refers to the appearance of variants that are stable upon passage and lack expression of *vir*-regulated factors. Two spontaneously occurring types of mutation have been characterized that give rise to phase variants. One is a frameshift mutation within a string of C residues which can be observed to revert to vir^+ (13). The other is the occurrence of small deletions internal to the *vir* locus which presumably are not reverting (8). The significance of either of these mutations to the biology and natural history of *B. pertussis* and other *Bordetellae* is not clear.

The *vir* locus has been cloned and shown to regulate the expression of two *B. pertussis* loci cloned in *Escherichia coli.* One such locus is *fha* which encodes the information necessary for FHA synthesis (11, 15). This locus is transcribed divergently from the *vir* locus. The other locus regulated by *vir* in *E. coli* is *vir* itself (10). To date, efforts by many researchers to demonstrate the regulation of other *vir*-regulated genes in *E. coli* have been unsuccessful, leading to the hypothesis that other regulatory factors are required in addition to *vir*-encoded factors to fully constitute *vir*-dependent regulation of virulence determinants. Based on the ability to stimulate the expression of FHA in *E. coli*, the extent of sequences encoding *vir* was mapped using Tn*5* mutagenesis. Based on this mapping and data presented herein, the *vir* locus was localized to a region of approximately 6 kilobases (kb). The DNA sequence of this region has been determined (1).

The DNA sequence of the *vir* locus predicts the presence of three open reading frames which have been called *bvgA*, *bvgB*, and *bvgC*. Interestingly, and in keeping with the environmental responsiveness of the *vir* system, two of the putative gene products, BvgA and BvgC, show sequence similarity with a family of bacterial regulatory proteins known collectively as two-component systems (9). One of the two components, termed the sensor, typically is a transmembrane protein capable of sensing environmental signals in the periplasmic space and communicating them to the interior of the cell. The portion conserved among members of the sensor class is in the carboxy-terminal portion of the protein. In all cases where it has been examined, the sensor protein has been shown to be a kinase, capable of autophosphorylation at a conserved histidine residue and subsequent transfer of the phosphate group (3).

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* Presenting author.

The acceptor of the phosphate group is the second component, termed the regulator protein. Among members of this class, the amino-terminal sequences are conserved; it is within this conserved domain that a phosphate group is accepted, at a conserved aspartate residue, from the sensor protein (3). Members of the regulator class are typically site-specific DNA-binding proteins which can thus positively and negatively regulate specific genes in response to specific signals transduced by the sensor protein. In the *vir* system, BvgA has N-terminal sequence similarity with the regulator class, and there is very good evidence supporting its role as a site-specific DNA-binding protein (C. Roy, personal communication). BvgC has sequence homology with the sensor class and is predicted to be a transmembrane protein with the amino-terminal 239 amino acids periplasmic, residues 240 to 261 making up a transmembrane segment, and the remaining 676 amino acids, including the sensor domain, cytoplasmic. Interestingly, BvgC also has a regulator domain carboxy-terminal to the sensor domain, the function of which is not understood. BvgB was predicted to be a periplasmic protein with homology to the amino-terminal portion of BvgC.

This paper presents a variety of genetic data accumulated over the past years. As stated earlier, some of these data have been used as the basis for determining the region to be subjected to DNA sequence analysis. Other data are being used to unravel the mechanisms by which the *vir*-encoded proteins regulate the virulence factors of *B. pertussis*.

MATERIALS AND METHODS

Bacterial strains. *B. pertussis* strain BP536 is a spontaneously occuring streptomycin-resistant mutant of BP338. This strain was the parent for all mutant strains constructed in this study. *E. coli* strain DH5α was used as the recipient for transformation in all cloning experiments and was purchased from Bethesda Research Laboratories. Constructs to be transferred to BP536 were then transferred to *E. coli* SM10 (12) for mating with *B. pertussis*. Conditions for mating have been described previously (14). *B. pertussis* strains were grown on Bordet-Gengou agar (Difco Laboratories) supplemented with 1% (wt/vol) proteose peptone (Difco) and 15% sterile defibrinated sheep blood. *E. coli* strains were grown on L agar. Both media were supplemented with antibiotics as needed.

Construction of kanamycin resistance gene insertions. The source of the kanamycin resistance gene insertion in all constructions was pKanπ (2). The kanamycin resistance gene fragment can be cut from this plasmid as a *Bam*HI, *Eco*RI, *Sal*I, or *Pst*I fragment. The plasmid pSS808, which is pRTP1 (14) containing a 14.7-kb *Bam*HI fragment containing the entire *vir* locus and part of the *fhaB* gene, was used in the construction of insertions at *Bgl*II, *Eco*RI, *Pst*I, *Sal*I, and *Xho*I sites. For *Bgl*II insertions, pSS808 was digested completely with *Bgl*II and ligated to the kanamycin resistance gene as a *Bam*HI fragment. For insertions at other sites, pSS808 was partially digested and then ligated to the kanamycin resistance gene as the corresponding fragment (*Sal*I cut pKanπ was used for *Xho*I insertions). These ligations were used for transformation of DH5α with subsequent selection for kanamycin resistance. In the case of *Eco*RI and *Xho*I insertions, some of the resulting plasmids had deletions of one or more fragments. These deletions were also included in the analysis.

For insertions at *Bst*BI, *Mlu*I, and *Pst*I sites, the plasmids containing linker insertions at these sites (see below) were used to generate the corresponding kanamycin resistance gene insertion by cutting in the middle of the linker with *Xho*I and inserting the pKanπ fragment as a *Sal*I piece. All insertions were transformed into SM10 and conjugated into *B. pertussis* selecting for kanamycin resistance. Kanamycin-resistant exconjugants were then streaked onto Bordet-Gengou plates containing kanamycin and streptomycin. Vir$^-$ colonies arising on these plates represented the desired insertion derivatives.

Construction of linker insertion mutations. For linker insertions at *Sal*I sites, an *Eco*RI fragment containing a kanamycin resistance gene insertion at the desired site was first subcloned from the derivatives constructed as described above into pBR322. The resulting plasmid was partially digested with *Pst*I and religated under dilute conditions. Amp^r, kan^s derivatives were screened by *Pst*I digestion of minipreps for those which had lost just the kanamycin resistance gene fragment. Since the polylinker flanking this gene has *Pst*I sites 6 base pairs (bp) inside the *Sal*I sites, this results in a plasmid with a 12-bp insertion in place of the kanamycin resistance gene insertion.

For linker insertions at *Bst*BI, *Mlu*I, and *Pst*I sites, an *Eco*RI fragment containing either the left or right half of the *vir* locus (Fig. 1) was cloned into pBR322. The resulting plasmids were digested partially with *Bst*BI, *Mlu*I, or *Pst*I, treated with calf intestinal alkaline phosphatase, and ligated in the presence of phosphorylated synthetic 12-bp oligonucleotide linkers. These linkers corresponded to an *Xho*I site flanked by the half-site created by digestion with the desired enzymes. Colonies

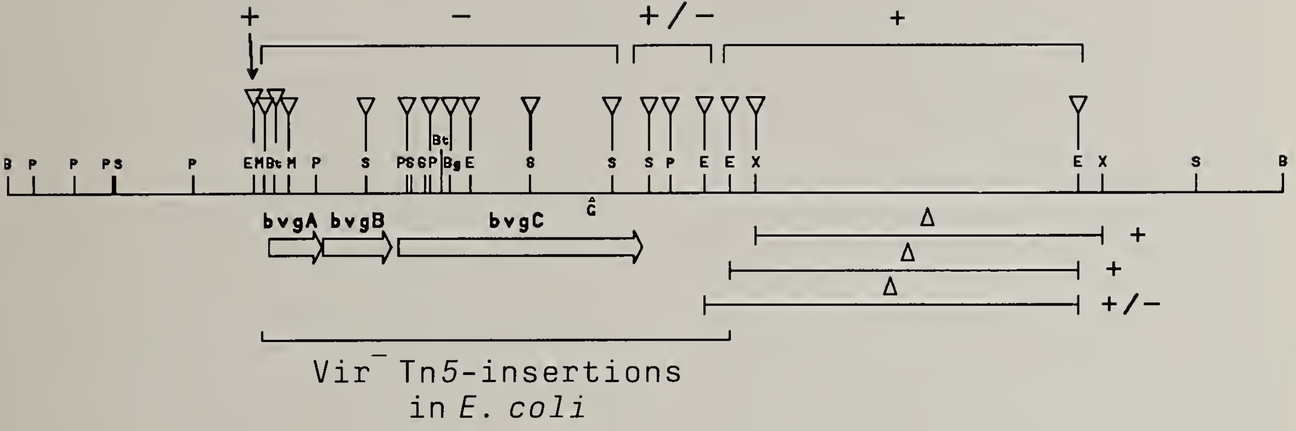

+
−
+/−
+
B P P PS P EMBtM P S PSBP Bt Bg E B S S P E E X E X S B
bvgA bvgB bvgC
Δ
+
+/−
Vir⁻ Tn5-insertions in E. coli

FIG. 1. A map of the 14.7-kb *Bam* HI fragment containing the *vir* locus. Positions of kanamycin insertion mutations which have been returned to the *B. pertussis* chromosome are shown above the map. Abbreviations for restriction sites are: B, *Bam* HI; Bt, *Bst* BI; E, *Eco* RI; M, *Mlu* I; P, *Pst* I; S, *Sal* I; and X, *Xho* I. Insertions which had accompanying deletions are shown below the map as bars marked with a Δ. The phenotype of the mutations are shown above the map as +, +/−, and −, and are explained in the text. The position of the predicted open reading frames corresponding to *bvgA*, *bvgB*, *bvgC*, and the region defined by Tn*5*-insertions in *E. coli* (15) are shown for reference.

arising were screened for the introduction of an *Xho*I site at the appropriate location, and possible multiple insertions of the linker were subsequently removed by digestion with *Xho*I and religation.

The *Eco*RI fragments containing the introduced mutations were recloned into pSS1129 which is pRTP1 containing a gentamicin resistance gene. After transfer into SM10 and conjugation with BP536, gentamicin-resistant derivatives were obtained. These derivatives were then streaked onto Bordet-Gengou agar containing streptomycin to select for loss of the plasmid from the chromosome. Vir⁻ derivatives represented the desired linker insertion mutants.

Homogenotization. To transfer the linker insertions onto a plasmid for use in a complementation assay, pSS528 was introduced by conjugation into the linker insertion mutant strains. The plasmid pSS528 consists of the broad host-range plasmid pRK290 (5) containing the 14.7-kb *Bam*HI fragment containing the *vir* locus (15). Such strains were Vir⁺ in phenotype due to complementation of the chromosomal mutation by the wild-type *vir* locus in *trans*. When these merodiploid strains were maintained on Bordet-Gengou agar containing tetracycline to maintain selection for pSS528, Vir⁻ derivatives would arise. These derivatives represented the result of recombination between the chromosomal and plasmid alleles such that the mutation was now incorporated into the plasmid. Plasmid DNA was isolated from these derivatives and used to transform *E. coli* DH5α. The plasmids recovered showed the restriction digest patterns predicted for the insertion of an *Xho*I site at the expected location. These plasmids were transformed into SM10 for conjugation back into the different linker insertion mutant strains.

RESULTS

The *vir* locus has previously been mapped in *E. coli* based on its ability to stimulate expression of FHA. Such mapping has two potential problems. One problem is that the *vir* locus may function differently in the *E. coli* background than it does in *B. pertussis*. Some factors that may be necessary for correct functioning may be missing, while other inhibiting factors may be present. Another problem is that the *vir* locus is by definition pleiotropic in its effects, and mapping based on effects on only one gene could give an incomplete picture. To alleviate these problems, we set out to map the *vir* locus in *B. pertussis* itself.

The strategy used depended on the ability to create, in vitro, mutations in the *vir* locus and return those to the *B. pertussis* chromosome to replace the wild-type locus. This task was accomplished through the use of a vector called pRTP1 (14). This vector can be used to select two consecutive genetic crossovers between a cloned gene and its chromosomal counterpart, thus replacing part or all of the chromosomal gene. Selection for the first crossover corresponds to the selection for maintenance of the incoming plasmid (introduced by conjugation) by chromosomal integration. This is a selectable event because (i) the vector is incapable of replicating in *B. pertussis*, and (ii) it encodes antibiotic resistance genes.

Once integrated, a second crossover is selected for by imposing selection for the loss of the vector sequences. This is accomplished through the action of the *rpsL* gene of *E. coli* which is present on pRTP1. This gene confers sensitivity to the antibiotic streptomycin upon a bacterium which is normally streptomycin resistant due to a chromosomal mutation in the homologous gene. Thus, those bacteria surviving streptomycin selection are those which have lost the vector sequences via a second crossover. If the mutation being introduced encodes a selectable phenotype such as kanamycin resistance, maintaining selection for that marker throughout the above scheme ensures that only the desired replacement derivatives will emerge in the final step.

The *vir* locus was cloned into pRTP1 on a 14.7-kb *Bam*HI fragment. Subsequently, insertions of a kanamycin resistance gene were introduced at restriction sites in the *vir* locus. Insertions at *Sal*I, *Pst*I, *Eco*RI, *Xho*I, *Bgl*II, *Mlu*I, and *Bst*B1 sites were created by partial or complete digestion and ligation to the inserted fragment. Some deletions were also created by this process. These insertion and deletion mutations have been returned to the *B. pertussis* chromosome as described above.

Figure 1 shows the positions of mutations which have been introduced along with their Vir phenotypes. The Vir phenotypes given in this figure were originally based on the degree of hemolysis on Bordet-Gengou agar, an assay which should indicate the levels of AC/Hly present. Thus, those mutations labeled "–" were nonhemolytic; those labeled "+" were as hemolytic as wild type; and those labeled "+/–" had an intermediate phenotype. These strains were also assayed by Western blot for the synthesis of FHA, PT, and Ptn. All nonhemolytic strains were completely devoid of expression of these three virulence factors as well, whereas those with an intermediate or wild-type hemolysis phenotype synthesized all factors assayed. The region defined by insertions and deletions with a negative phenotype corresponds well with the DNA sequence data in that all of these mutations affect sequences within or just upstream of *bvgA*, *bvgB*, or *bvgC*. The region encompassing both "–" and "+/–" insertions corresponds to the mapping data obtained in *E. coli*.

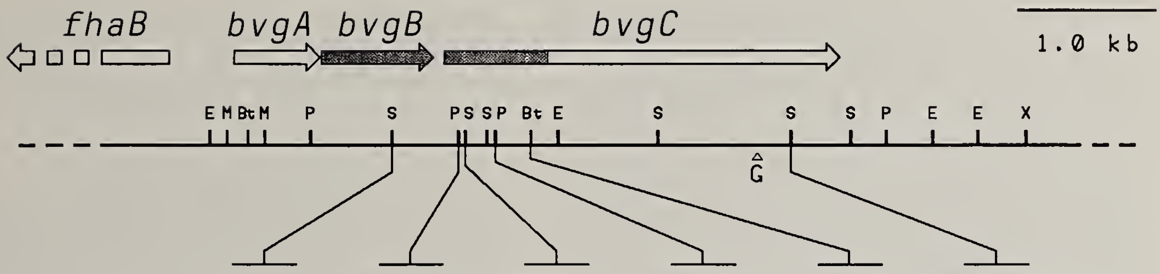
A complementation analysis was undertaken to further subdivide the *vir* locus into separate genes, or, more precisely, into genetic complementation groups. Such an analysis required a set of mutations throughout the *vir* locus which were nonpolar and which could be present on either of two replicons in a particular strain. The mutations were constructed by inserting 12-bp synthetic oligonucleotide linkers at restriction sites. Such mutations cause a net insertion of four amino acids in the encoded protein, producing a lesion expected to abolish activity in most cases, but not to cause a polar phenotype. The mutations were crossed into the *B. pertussis* chromosome using a derivative of pRTP1 called pSS1129 which includes a gene for gentamicin resistance. This provides an improvement over ampicillin resistance for the selection for the first crossover in cases where the mutant allele lacks a selectable marker such as kanamycin resistance. To allow the pair-wise testing of these mutations, each was crossed from the chromosome of *B. pertussis* onto the plasmid pSS528 through the process of homogenotization as described above. Each of the resulting mutant plasmids could then be tested in all the mutant strains for its ability to complement and restore a Vir^+ phenotype.

Before this analysis was completed, the DNA sequence of the *vir* locus became available and suggested the presence of three genes *bvgA*, *bvgB*, and *bvgC* (Fig. 1 and 2). Linker insertion mutations within the regions predicted to code for BvgB and BvgC were all Vir^-. However, three different mutations within the region predicted to code for BvgA, i.e., at the *Pst*I, *Mlu*I, and *Bst*BI sites, all retained partial or complete activity.

All of the mutations with a negative phenotype were tested in pair-wise combinations and against the wild type. It can be seen that this analysis (Fig. 2) failed to show that mutations in *bvgB* complement those in *bvgC* and vice versa. Posssible reasons for this and implications of these data are discussed below.

DISCUSSION

The *vir* locus was mapped by examining expression of the virulence-associated factors FHA, AC/Hly, PT, and pertactin in strains of *B. pertussis* containing insertions (or insertions with accompanying deletions) in or near the *vir* locus. Mutations that affected *vir* activity fell into two classes: those that completely abolished activity and those that only slightly reduced activity. In no case did any of the mutations knock out only one or a subset of the assayed virulence determinants. Thus, it does not appear to be the case that *vir* acts at multiple loci because



Plasmid:

Chromosome	729	789	742	788	840	739
729	−	−	−	−	−	+
789	−	−	−	−	−	+
742	−	−	−	−	−	+
788	−	−	−	−	−	+
840	−	−	−	−	−	+
739	−	−	−	+	−	−
536(wt)	−	−	−	+	−	+

FIG. 2. Results of a genetic complementation assay. The position of linker insertion mutations introduced into the *vir* locus are shown in relationship to the restriction map of *vir* and the *bvg* genes, and are identified by number. Regions of the *bvg* genes predicted to encode periplasmic protein sequences are shaded (1). The mutations were tested in pair-wise combinations with one allele chromosomal and one present on the plasmid pSS528. This plasmid is of the IncP incompatibility group, replicates in *B. pertussis*, and is expected to have a copy number of 5 to 7 per chromosome. The hemolytic phenotype observed on Bordet-Gengou agar is shown by a + or −.

it encodes multiple independent regulatory elements, but rather that all elements encoded within the *vir* locus act in concert by a similar mechanism to regulate all of the virulence determinants.

This is not to say that *vir* acts directly at all of these regulated genes. In fact, two lines of evidence suggest that this is not the case, since (i) virulence determinants other than *fha* are not regulated by *vir* when cloned into *E. coli* in the presence of *vir*, and (ii) the upstream regions expected to contain positive regulatory sites do not show obvious similarities when compared among genes for virulence determinants. Thus, there must be other regulatory factors that are themselves regulated by *vir* and, in turn either regulate the other virulence determinants or act in concert with *vir*-encoded proteins to regulate the other determinants. The genes for these co-regulators should be identifiable by mutations which affect only one or a subset of virulence determinants.

The region defined by those mutations which completely abolish activity corresponds very well to the region predicted by DNA sequence analysis to contain the genes *bvgA*, *bvgB*, and *bvgC*. The region downstream of the predicted *bvgC* gene contains sites where insertions in *B. pertussis* have a slightly, but reproducibly, reduced hemolytic phenotype. The fact that these mutant strains maintain some ability to synthesize four of the major virulence determinants indicates that this region is not absolutely required for *vir* activity. However, Tn*5*-insertion mutations in this region in *E. coli* were reported to be *vir*⁻ on the basis of their effect on FHA synthesis (15). Therefore, insertion mutations in this region might affect the level of *vir* activity but not its nature. This could be due to an effect of these insertions on the stability of mRNA-encoding Bvg proteins. The apparently greater effect of these mutations in *E. coli* could be due either to a greater instability of the message in this genetic background or to the effect of Tn*5*-insertions as opposed to the insertions constructed in *B. pertussis.*

Attempts to demonstrate genetically the presence of multiple, complementing genetic units within the *vir* locus failed. However, the results obtained, when interpreted in the context of the DNA sequence analysis, allow for some interesting observations. Mutations in *bvgA* were excluded from the complementation assay because none were completely negative. The assay failed to show that *bvgB* and *bvgC* are separate genes. There are at least three confounding factors or potential bases for this phenomenon, the most persuasive of which is that the one mutation in *bvgB* (729) and three of four mutations in the part of *bvgC* encoding the periplasmic domain (742, 789, 840) have a negative phenotype that is *trans* dominant over the wild-type. In other words, when the mutant allele is present on the plasmid in multicopy over the wild type chromosomal allele, a negative phenotype results. This result makes it impossible to see complementation between the gene containing this mutation and any other. One of the simplest means of explaining this result is to hypothesize that BvgB and BvgC normally function in a multimeric state. Thus, an excess of a mutant protein results in the formation of an even greater excess of multimers which contain at least one mutant protein and are therefore nonfunctional. In the reverse situation, when the wild type is in multicopy over the mutant allele, a wild-type phenotype results (data not shown), in keeping with such a model.

Other explanations of these results are, of course, possible, but a second observation provides support for the multimerization hypothesis. This observation is that intracistronic complementation can occur. The one mutation affecting the periplasmic domain of BvgC which is not *trans* dominant over wild type (788) will complement a mutation much later in the *bvgC* gene (739) and vice-versa. Thus, two mutant BvgC proteins—one affected in the periplasmic N terminus of BvgC and one in the cytoplasmic C terminus—can, although nonfunctional by themselves, function together to activate a *vir*-regulated gene. It is difficult to envision how this complementation could occur in the absence of direct interaction of the BvgC molecules: this represents the second of the confounding factors in that the simplest genetic interpretation of such a result would be that two genes were present, when in fact there is only one.

A third reason the complementation analysis failed to show, that *bvgB* and *bvgC* are separate genes is that, in fact, they are not separate. Recent results, which will be presented elsewhere, have demonstrated conclusively that BvgB and BvgC are made as one protein, called BvgS (for sensor), of a size consistent with the sum of their predicted sizes. This result was not accessible in the genetic analysis due to the *trans* dominance of the mutations used. The finding simplifies the analysis a great deal, however, because models invoked to explain the phenotype of these mutations do not need to invoke BvgB multimers plus BvgC multimers, or perhaps mixed multimers of BvgB and BvgC, but rather just multimers of BvgS. Thus, of five mutations constructed which affect the periplasmic domain(s) of BvgS, four have a *trans*-dominant effect over wild type, and one is capable of intracistronic complementation.

The genetic studies described here provide an unforeseen clue to the mechanism of signal transduction in the *vir* system of virulence regulation in *B. pertussis.* Two lines of genetic evidence, the *trans* dominance of mutations in the periplasmic domain of the sensor protein BvgS and intracistronic complementation of *bvgS* alleles in different domains, suggest that this protein is functional as a multimer. Such a model provides an explanation for how signals could be transduced from the exterior to the interior of the bacterial cell. In a simple form of this model, dimerization of BvgS would be mediated by interactions of the periplasmic domain and would be sensitive to modulating conditions (e.g., $MgSO_4$). Activation of BvgA in the cytoplasm would be dependent on the dimeric state of the C-terminal sequences of BvgS. Thus, in the presence of external modulating conditions, the internal dimeric state of BvgS is disrupted and BvgA is not activated. The dependence of the ability of BvgS to activate BvgA on dimerization can be explained in several ways. An intriguing possibility is suggested by the presence of the "extra" regulator domain of BvgS. Phosphorylation of this domain would be required for activity, and a monomeric BvgS molecule (present under modulating conditions) would be incapable of autophosphorylation. When dimers are formed, however, as in nonmodulating conditions, the sensor or kinase domain of one BvgS molecule could phosphorylate the regulator domain of a neighboring BvgS molecule, thus activating BvgA. Models such as this provide predictions regarding the behavior of *vir* components can be tested genetically as well as biochemically as the necessary tools evolve. Such experiments are under way.

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DISCUSSION

DR. ULLMANN: (Question inaudible).

DR. STIBITZ: The S protein or the A protein?

DR. ULLMANN: The A protein.

DR. STIBITZ: It has not been identified directly in BvgA but there is good homology with the phosphate acceptor site common on the proteins.

DR. ULLMANN: Yes, and did you use . . . (inaudible).

DR. STIBITZ: In BvgA it is an aspartate residue. Those experiments are definitely on the books but with the impending furlough, I am not sure when we will get to them.

DR. LOCHT: Are there any mutants known in the periplasmic domain of Bvg5?

DR. STIBITZ: (inaudible) . . . that are mod mutations? They would be constitutive. They are not but that would be a class of mutations that we are going to try and look for and I think it will be very interesting.

DR. BAILEY: I was wondering if you know by your antibodies whether or not the N terminus is linked to the membrane or if it is free?

DR. STIBITZ: If you mean by looking in the protoplasts or something like that, we have not had a chance to do those experiments yet.

DR. RAPPUOLI: First, I would like to congratulate you on a very nice piece of work. And, it is good that you found the mistake in the sequence, we are very glad about that. Secondly, I think we need to think about the regulator domain of the sensor protein. What role is this part of the protein playing and why is it necessary for *vir* activity? Do you have any comments about that?

DR. STIBITZ: We have tried to incorporate that in our model but as you say, at this point the evidence is just that mutations within that domain totally abolish *vir* activation. In terms of experiments to look at whether it is phosphorylated, the experiments which Agnes suggested are something we would definitely like to do. The phosphate acceptor site in that regulator domain is just as highly conserved as it is in the bvgA protein so we would expect that it is also a phosphate acceptor site, but we have not had a chance to do those experiments yet.

DR. RAPPUOLI: I would also like to ask you what you feel about the importance of temperature in modulation?

DR. STIBITZ: My personal feeling is it is true that it is easiest to invoke a role for temperature in a biological sense because that is the only signal which we associate with the human host. On the other hand, and I think Angela Melton has looked at this much more carefully than I have, temperature does not seem to be as tight a regulation through *vir* as the magnesium sulphate and nicotinic acid. In other words, you have to drop the temperature quite low to get a totally *vir* minus organism and if *Bordetella* really is only found in the human host, the small variations in temperature which are cited at different points in the respiratory tract, do not seem to be enough in the laboratory to cause a major difference in regulation. So the short answer is, I do not know.

DR. RAPPUOLI: The reason I ask is that I think perhaps we can think about vir-regulation originally evolving in *B. bronchiseptica*. This would fit in with the observation that *B. bronchiseptica* strains may survive in the environment and perhaps the first role of vir was in *B. bronchiseptica* to sense the difference between being in the host and being in the environment, using temperature as a cue.

DR. STIBITZ: Is that right? I was not aware that *B. bronchiseptica* could be isolated from non-host-associated sources.

DR. RAPPUOLI: I am not aware myself of a report that *B. bronchiseptica* has been isolated from environmental sources, however, given the fact that this bacterium can survive and grow in very simple media such as marine water, I believe that it could be present in the environment. We should also keep in mind that *B. bronchiseptica* is the only species of *Bordetella* which is motile. This suggests an environmental origin of this pathogen which may also explain the evolution of the vir system.

DR. STIBITZ: I agree completely. That would explain it very nicely.

DR. KNIGHT: When you showed your slides on the *E. coli* fusions, you . . . (inaudible).

DR. STIBITZ: That is because the N-terminal start for the *bvgC* fusions was defined by our PCR primer and was picked based on the DNA sequence. In other words, it started where we told it to, not where we would have allowed it to naturally, and that is why we got those results.

DR. ROY: I like the dimerization model but the only problem that we see with it is that a number of these other sensor . . . (inaudible) . . . yet all the . . . (inaudible) . . . which have been isolated and a number of them have been characterized seem to be . . . (inaudible). And you get a dimerization that . . . (inaudible) . . . key to the interaction that you can isolate these which are constitutively dimerized and it would be local like that periplasmic region.

DR. STIBITZ: There are a couple of answers. One may be that it is easier to functionally get those in linker region. The other thing is that we are beginning cross-linking experiments now to look at the state of dimerization and I think what is going to clear up a lot of questions is to correlate the biochemical phenotype with the genetic phenotype and the mod mutations would be ones that I would be especially interested in looking at to see if those, for example, remain dimerized even under modulating conditions.

DR. WARDLAW: (Comment inaudible).

DR. STIBITZ: I think those are very interesting observations and I have definitely learned something here today. If this is true, it would make the *vir* locus an even closer counterpart to *toxR* of *Vibria cholerae.*

Regulation and Functional Characterization of *Bordetella pertussis* Adenylate Cyclase-Hemolysin

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Adenylate cyclase (AC) and hemolysin (Hly) are major virulence factors produced by *Bordetella pertussis* (27). The cloning and sequencing of the structural gene, *cyaA*, revealed an open reading frame of 1,706 codons (3) encoding a single bifunctional protein (CyaA protein, or cyclolysin) of approximately 200 kDa endowed with both AC and hemolytic activities (4). The 400-residue amino-terminal domain corresponds to the calmodulin-(CaM) activated catalytic region whereas the carboxy-terminal 1,300 residues, showing 25% similarity with *Escherichia coli* alpha hemolysin, are responsible for the hemolytic activity of *B. pertussis.* The *cya* operon is composed of four genes: the structural gene *cyaA* and three genes — *cyaB, D,* and *E* — necessary for the secretion of the CyaA protein and located downstream from the *cyaA* gene (4).

The structural organization of the CaM-activated catalytic moiety of the 43-kDa protein was determined using biochemical and genetic approaches (2, 9, 11); and led to the identification of the catalytic and CaM-binding domains of *B. pertussis* AC. The AC enzyme and toxin activities have been studied by several investigators who have ascribed these activities to different forms of the protein. It now appears, however, that the 200-kDa CyaA protein represents the AC toxin molecule (7, 21; J. Bellalou, H. Sakamoto, D. Ladant, C. Geoffroy, and A. Ullmann, Infect. Immun., in press).

The expression of virulence-associated genes in *B. pertussis* is coordinately regulated by the gene products encoded by the *bvg* locus (13); however, the molecular mechanisms of this regulation are far from clear. Based on recent data reported by Miller et al. (14) and Roy et al. (22), it seems likely that different regulatory mechanisms are involved in the transcriptional activation of individual virulence determinants.

This report presents studies on the relationship between the structure and functions of the CyaA protein, with special emphasis on its secretion, pore formation, and entry into target cells. Data are also provided on the genetic and environmental regulation of the *cya* operon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bordetella* strains were grown on Bordet-Gengou agar containing 15% sheep blood; X-mode cultures, grown in Stainer and Scholte medium (SSM) (25) were then supplemented with 2mg/ml of bovine serum albumin (BSA). All media were sterilized on a 0.22-µm Sterivex filter (Millipore Corp.). Growth was carried out at 36°C under high-rate aeration. C-mode cells, characterized by their loss of virulence factors, were grown in SSM containing 4 mM nicotinic acid. *E. coli* strains were grown in LB medium (15) supplemented with the appropriate antibiotics, when necessary.

Recombinant DNA methods. Standard methods were used for isolating and transforming plasmid DNA, restriction enzyme digestions, and DNA ligations.

Construction of deletion strains. Strains 18HS2, 18HS3, and 18HS4 were constructed by replacing chromosomal *cya* DNA sequences of strain 18323 by double recombination events, using plasmid pRTP1 (26) generously provided by S. Falkow. After the entire *cya* operon was inserted into plasmid pRTP1, internal deletions were generated which led to either the excision of most of the *cya* operon (*Bam*HI deletion of 10,000) or to in-phase deletions in the *cyaA* gene (*Bgl*II and *Cla*I deletions of 468 and 177 bp, respectively). The resulting strain 18HS2 carried the large *Bam*HI deletion; strains 18HS3 and 18HS4 carried the *Bgl*II and *Cla*I in-phase deletions, respectively.

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Purification of the 200-kDa AC (CyaA protein). Extracellular CyaA protein and cell-associated CyaA proteins were purified as described previously (Bellalou, Sakamoto, Ladant, Geoffroy, and Ullmann, in press). The specific activity of the purified proteins was 400 µmol cAMP/min/mg protein.

Assay of enzymatic activities. AC activity was determined in the presence of 0.1 µM of CaM as described previously (9). One unit of enzyme corresponds to 1 µmol of cAMP formed per min at 30°C and pH 8. ß-galactosidase activity was measured according to Pardee et al. (19). One unit of enzyme corresponds to 1 nmol substrate formed per min at 28°C. ß-lactamase was assayed as described by O'Callaghan et al. (18). One unit of enzyme corresponds to 1 nmol nitrocefin hydrolyzed per min at 28°C.

Assay of hemolytic activity. Serial dilutions of crude preparations or purified fractions in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$ were mixed with an equal volume of 1% sheep erythrocyte suspension (3 x 10^8 cells/ml), suspended in the same buffer, and incubated 18 h at 37°C. Samples were centrifuged to remove unlysed erythrocytes, and supernatant absorbance was measured ($\lambda = 541$ nm) to determine the percentage of erythrocyte lysis.

Determination of AC toxin capacity. Sheep erythrocytes (2 x 10^8 cells) were incubated with purified CyaA protein at 37°C or 4°C in 0.2 ml TS buffer (10 mM Tris-HCl pH 7.5, 0.15 M NaCl) containing 2 mM of either $CaCl_2$ or EDTA. At different time intervals, samples were diluted in 2 ml cold TSE buffer (TS buffer supplemented with 2 mM of EDTA) and centrifuged 5 min at 3,000 x g at 4°C. Cell pellets were resuspended in 0.25 ml TSE. This suspension was used to determine (i) intracellular cAMP accumulation and (ii) AC internalization as described previously (Bellalou, Sakamoto, Ladant, Geoffroy, and Ullmann, in press).

Immunoblotting. Proteins separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis were transferred to nitrocellulose sheets and incubated with anti-AC rabbit immune sera (17). The immunochemical detection was performed with alkaline phosphatase-labeled anti-rabbit immunoglobulins.

RNA isolation and primer extension analysis. RNA was isolated as described previously (12). For primer extension analysis, 0.07 pmol of [γ^{32}P]ATP-labeled oligonucleotides was annealed with 50 µg of total RNA by precipitating the DNA and RNA with ethanol, resuspending the pellet in 5 µl H_2O, and then heating the mixture to 100°C for 5 min and allowing the temperature to drop slowly to 42°C. A 1:10 volume of 10 x avian myeloblastosis virus (AMV) buffer (10 x buffer:50 mM Tris-HCl pH 8.3, 500 mM KCl, 100 mM $MgCl_2$, 10 mM DTT), 0.1 mM final concentration deoxynucleoside triphosphates (dNTPs), and 9 U of AMV reverse transcriptase were added and the reaction carried out at 42°C for 30 min. One µg/ml of RNase A was then added, and incubation continued for another 15 min. Next, 7 µl formaldehyde stop buffer was added, and the samples were electrophoresed on 6% polyacrylamide-8M urea denaturing sequencing gels. Finally, 30-mer oligonucleotides with complementary sequence to the 5′ end of the *cyaA* and *cyaB* genes were synthesized, resulting in oligonucleotide A: GCGTAGTCCGACCAATGCGTTTGCG GCGGC and oligonucleotide B: GACGGACCAGTAC GACCGAGCGATAGTGCC.

DNA sequencing. DNA sequence ladders were prepared using the dideoxynucleotide method of Sanger et al. (23). Oligonucleotides A and B were used as primers for sequencing the 5′ ends of *cyaA* and *cyaB*, respectively.

RESULTS

Secretion of the CyaA protein. The *cyaA* gene product (CyaA protein) is synthetized as a 200- to 215-kDa protein. Low-molecular-weight forms of 43- to 50-kDa, exhibiting CaM-activated AC activity, were previously isolated from *B. pertussis* culture supernatants (6, 8, 9, 10, 24), suggesting that proteolytic processing was a prerequisite for secretion. We searched for high-molecular-weight forms of CyaA protein, present in crude supernatants of early exponential phase cultures, grown in the presence of BSA and CaM. As shown in Table 1, when BSA was present in the culture media, 12 to 45 times more AC was found in culture supernatants of several *Bordetella* species. When BSA and CaM were both present in the culture medium, two to three times higher AC activity could be detected in the supernatants. Fig. 1 shows a Western blot analysis of culture supernatants of *B. pertussis* strain 18323: anti-AC antibodies reacted with a major polypeptide of 200 kDa; the reaction was more prominent when bacteria were grown in the presence of BSA or BSA and CaM. While some degradation products also reacted with the antibodies, the 45-kDa form could barely be detected. The 200-kDa form, when submitted to limited proteolysis with trypsin, was converted into a 43- to 50-kDa protein with no loss of AC activity (1). These results show that the 200-kDa CyaA protein is secreted into the external medium without proteolytic processing. It is therefore likely that the

TABLE 1. Secretion of AC under different growth conditions[a]

Strain	AC activity in culture supernatants (nmol cAMP/min per ml)		
	SSM	SSM + BSA[b]	SSM + BSA + CaM[c]
B. pertussis (18323)	100	1,500	3,300
B. pertussis (Tohama)	2	50	150
B. bronchiseptica	60	720	ND
B. parapertussis	12	540	ND

[a] Cultures were grown 24 hours at 36°C.
[b] SSM with 2 mg/ml bovine serum albumin.
[c] SSM with 80 µg/ml calmodulin.
ND, not determined.

low-molecular-weight forms of AC purified from culture supernatants, result from proteolytic processing of the protein's secreted high-molecular form. The presence of BSA and CaM would protect the secreted CyaA protein from proteolytic degradation.

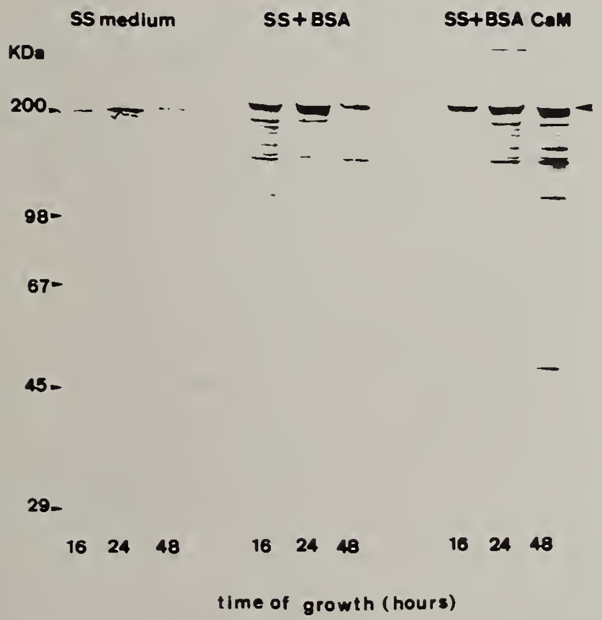

FIG. 1. Immunoblots of *B. pertussis* culture supernatants. Strain 18323 was grown in SSM in the presence of either 2 mg/ml of BSA or 2 mg/ml of BSA + 80 µg/ml CaM. Immunoblotting was performed as described in the text. Arrowheads indicate the 200-kDa CyaA protein.

Characterization of the hemolytic and toxin activities of the CyaA protein. Complementation studies of *B. pertussis* strains deficient in hemolytic activity have shown that the carboxy-terminal residues of the 200-kDa *cyaA* gene product are responsible for the hemolytic phenotype (4). Furthermore, Hewlett et al. (7) and Rogel et al. (21) have shown that the toxic form of AC corresponds to the 215-kDa CyaA protein. Having previously shown that the CyaA protein was secreted as a 200-kDa protein (1), it was important to characterize the hemolytic and toxin activities of the secreted CyaA protein and compare them with those of the cell-associated form. Fig. 2 shows the time- and concentration-dependent hemolytic activity of concentrated, cell-free *B. pertussis* supernatants incubated with sheep erythrocytes. The hemolytic activity of the CyaA protein is calcium-dependent. It is also at least 100 times lower than that of *E. coli* alpha hemolysin about 0.5 µg/ml of CyaA protein was required to obtain 100% lysis in 8 hours. To determine if the protein's weak hemolytic activity was due to some post-secretory modifications, we purified the 200-kDa CyaA protein from bacterial extracts and culture supernatants. As shown in Table 2 no significant difference could be found between the hemolytic activities of purified cell-associated or secreted CyaA proteins; moreover, AC and hemolytic activities were strictly correlated.

Purified CyaA proteins were also tested for their toxin activities by measuring in parallel AC internalization and

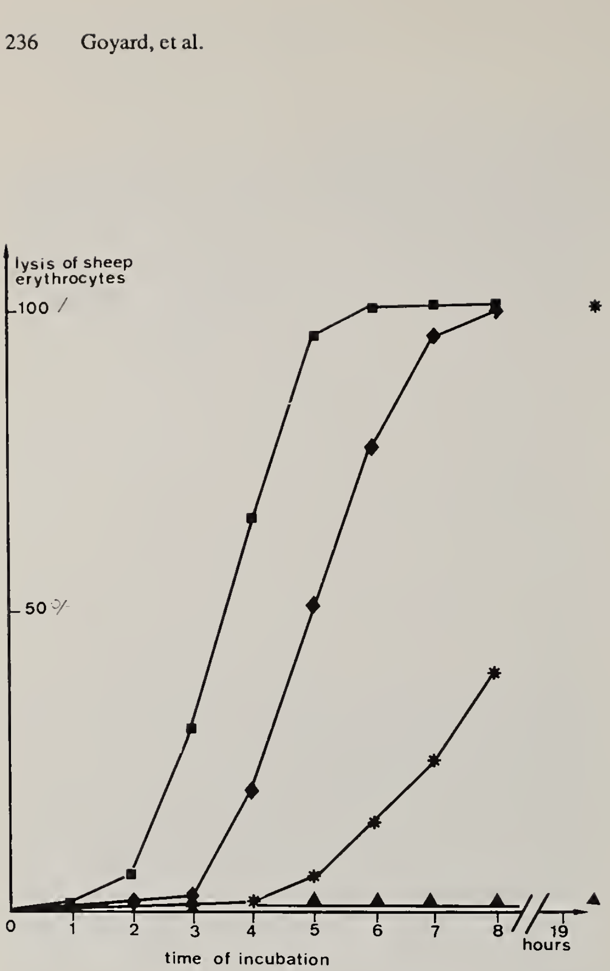

FIG. 2. Time- and concentration-dependent hemolytic activities of *B. pertussis* culture supernatants. Supernatants of mid-log phase cultures of strain 18323 grown in SSM supplemented with BSA were concentrated eightfold, and aliquots were incubated with sheep erythrocytes as described in the text. AC activities of the samples used in the hemolytic assays were: 4 (■), 2 (◆), and 1 (*) U/ml (▲ as ■ in the absence of calcium).

cAMP accumulation in sheep erythrocytes (Fig. 3). The secreted and cell-associated CyaA proteins displayed comparable toxin activities; both cAMP accumulation and AC internalization were calcium- and temperature-dependent. The catalytically active 45-kDa form of AC released by proteolytic digestion of the 200-kDa CyaA protein, showed no toxin activity. These results demonstrate that the secreted, catalytically active, 200-kDa CyaA protein exhibits both hemolytic and toxin activities.

To test whether the protein's structural integrity is required for hemolytic and toxin activities, we constructed an almost complete deletion of the *cya* operon (strain 18HS2) in *B. pertussis* strain 18323 and created two in-phase deletions in the *cyaA* gene. One of the deletions (strain 18HS3) removed 156 codons in the reading frame from Ile (623) to Glu (779); the second one (strain 18HS4) removed 59 codons from Ile (827) to Thr (886) in regions highly conserved between *B. pertussis* CyaA protein and *E. coli* Hly. The AC, hemolytic, and toxin activities of the corresponding wild-type and truncated CyaA proteins are summarized in Table 3. The data support the following conclusions: (i) the secretion of the truncated CyaA proteins was not impaired and their AC activities were comparable to that of the wild-type protein; and (ii) the hemolytic activity of the truncated CyaA proteins was severely reduced and their toxin activity abolished. These results demonstrate that structural integrity of the 200-kDa CyaA protein is required for toxin activity but not for hemolytic activity or secretory function.

Regulation of the *B. pertussis cya* operon. The *cya* operon of *B. pertussis* is composed of four open reading frames, *cyaABDE* of ≈10 kb in length (4). The *cyaBDE* genes are contiguous; there is a 77-bp intergenic region between *cyaA* and *cyaB*. To determine the transcriptional organization of the *cya* operon, Northern blotting experiments were used to characterize *cya*-specific in vivo mRNAs. We showed that in virulent *B. pertussis* strains, transcription initiated at the *cya* promoter produced an abundant *cyaA* species, but only low levels of

TABLE 2. Hemolytic activity of purified cell-associated and secreted CyaA proteins

Source of CyaA[a] protein	AC activity (µmoles/min per ml)	Hemolysin[b] % of lysis
Cell-associated	0.05	27
"	0.1	50
"	0.2	90
"	0.3	100
Supernatant	0.05	30
"	0.1	65
"	0.2	100
"	0.3	100

[a] Specific activity 400 µmoles cAMP/min/mg.

[b] Liquid assay with sheep erythrocytes (1.5 10^8 cells/ml) in the presence of 1 mM of $CaCl_2$.

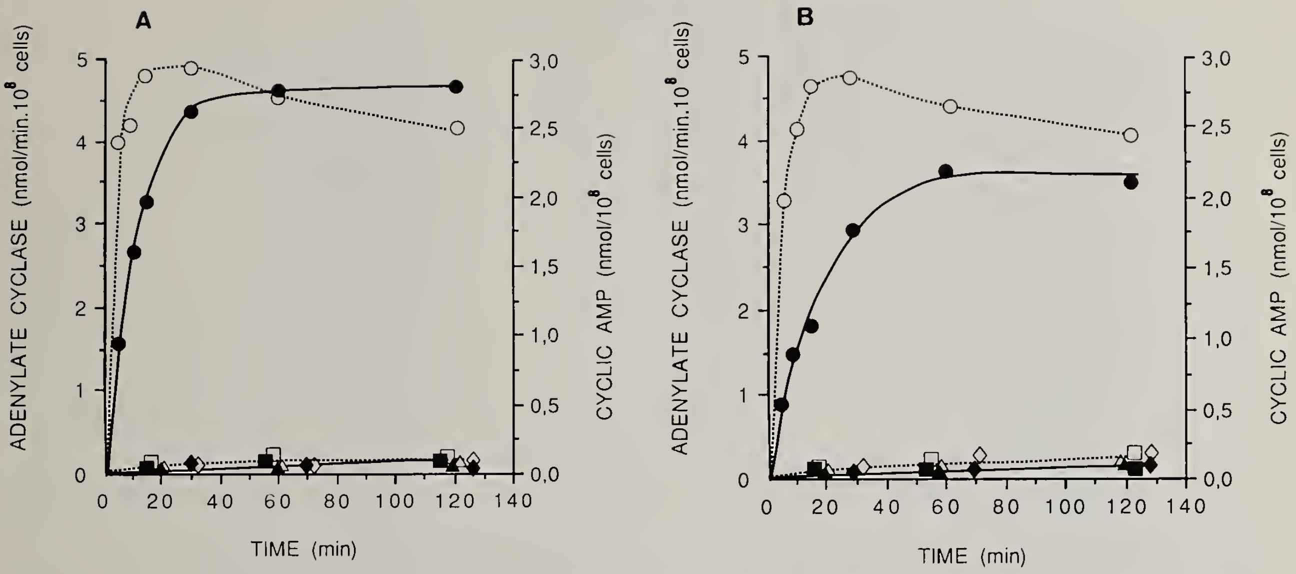

FIG. 3. Toxin activity of the CyaA protein. Purified preparations of secreted (panel A) and cell-associated (panel B) 200-kDa CyaA proteins, corresponding to 0.24 units of AC, were incubated (at time = 0 min) with sheep erythrocytes (2 x 10^8 cells) at 37°C (●, O, ▲, △) and at 4°C (■, □) in the presence of either 2 mM of Ca^{2+} (●, O, ■, □) or 2 mM of EDTA (▲, △). At the indicated times, intracellular AC activity (closed symbols) and cAMP (open symbols) were determined as described in the text. The toxin activity of the catalytically active 45-kDa fragment (♦, ◊) obtained by proteolytic digestion of the secreted (A) or cell-associated (B) CyaA protein was assayed at 37°C in the presence of 2 mM of Ca^{2+}. (Reproduced with the permission of American Society for Microbiology, Journals Division).

full-length *cyaABDE* transcripts. S1 mapping identified a transcription termination site 3′ from the *cyaA* gene within the *cyaA*-*cyaB* intergenic region (12). By primer extension analysis using an oligonucleotide complementary to the 5′ end of the *cyaA* mRNA, we localized the transcription start site of the *cya* operon. When *B. per-*

TABLE 3. AC, hemolysin, and toxin activities of *cyaA* mutants

Strain	Number of codons deleted in *cyaA*	AC activity[a] (nmol/min per ml)		Hemolytic activity [b] (%)	Toxin activity[c]	
		Cell-associated	Supernatant	Supernatant	cAMP accumulation	AC internalization
18323		150	440	100	3,500	250
18HS2	1,706	0	0	0	5	0
18HS3	156	100	330	12	69	0.9
18HS4	59	100	350	50	80	1.1

[a] Mid-log phase cultures of bacteria (between 1.5 x 10^{10} and 2 x 10^{10} bacteria/ml) grown in SSM supplemented with BSA were centrifuged. Cell pellets were resuspended in the same volume of 8 M urea in Tris-HCl 50 mM, pH8, CaCl$_2$ 0.2 mM. AC activities were determined in bacterial extracts and in the corresponding culture supernatants.

[b] The values were obtained from dose-response curves (% of hemolysis as a function of AC concentrations) performed with concentrated culture supernatants and represent hemolytic activities corresponding to 2 units of AC.

[c] Sheep erythrocytes were incubated for 1 h at 37°C in the presence of 2 mM of CaCl$_2$ with concentrated culture supernatants at a final concentration of 0.3 u/ml of AC. Intracellular cAMP is expressed as pmol/10^8 cells; internalized AC activity is expressed as pmol of cAMP/min/10^8 cells.

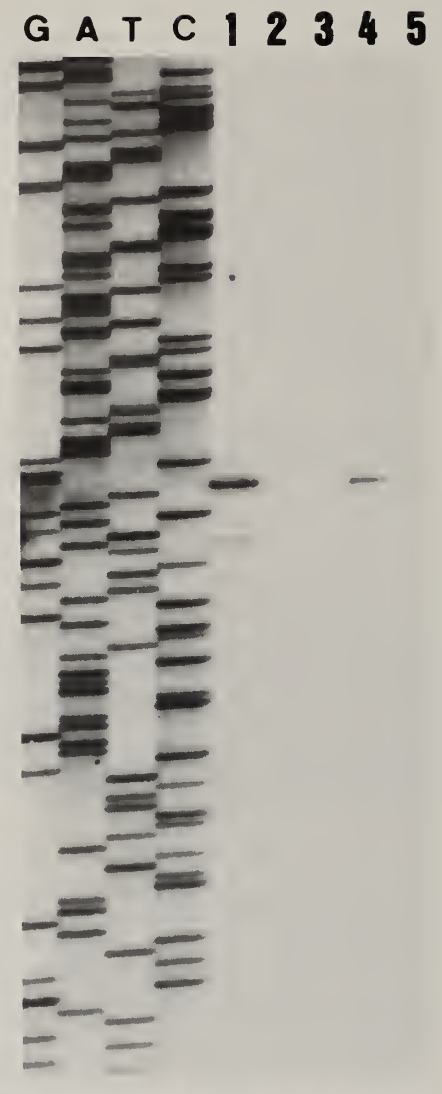

FIG. 4. Primer extension analysis of *B. pertussis* mRNA. Radiolabeled oligonucleotide A (see text) was hybridized to total RNA and primer extension was performed with AMV reverse transcriptase. RNAs from Vir+ (18323 Mod X, lane 1; Tohama I, Mod X, lane 4 and Vir⁻ strains (18323 Mod C, lane 2; BP347, lane 3; Tohama I, Mod C, lane 5) were used as templates. The same oligonucleotide was used as a primer for dideoxy sequencing; the sequence (of the noncoding strand) is shown. (Reproduced with the permission of EMBO Journal).

tussis strains were grown under nonmodulating (Mod X, Vir$^+$) conditions (Fig. 4, lanes 1 and 4), transcription starts at an A residue, located 115 bp upstream from the *cyaA* translational start codon, ATG. When RNA was isolated from strains grown under modulating (Mod C, Vir$^-$) conditions (Fig. 4, lanes 2 and 5) or from strain BP347 (29) which carries a Tn*5* insertion in the *bvg* operon (Fig. 4, lane 3), no primer extension products were found. Thus, *cyaA* is transcribed only in Mod X or *bvg*$^+$ *B. pertussis* strains.

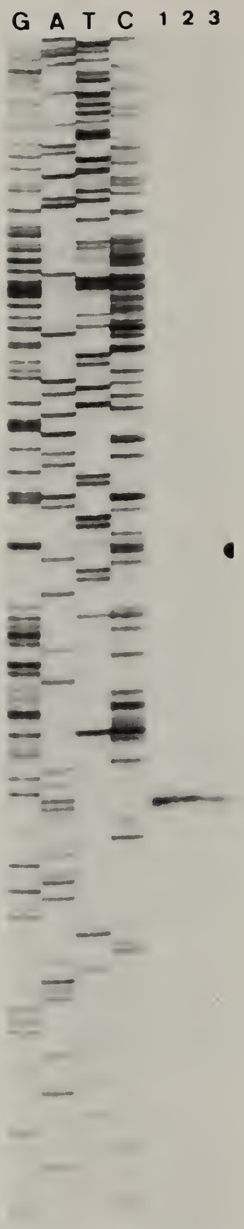

FIG. 5. Primer extension analysis of *B. pertussis* mRNA. Radiolabeled oligonucleotide B (see text) was hybridized to total RNA and primer extension was performed with AMV reverse transcriptase. RNAs from Vir$^+$ (18323 Mod X, lane 1) and Vir$^-$ strains (18323 Mod C, lane 2; BP347, lane 3) were used as templates. The same oligonucleotide was used as a primer for dideoxy sequencing; the sequence (of the noncoding strand) is shown.

TABLE 4. Expression of *cya::lac* fusions in *E. coli* and *B. pertussis*[a]

Host	Fusions	pLA5.7	β-galactosidase (U/mg protein)	β-lactamase (U/mg protein)	β-galactosidase / β-lactamase
E. coli	*cyaA::lacZY*	–	0.16	17,000	0.9×10^{-5}
E. coli	*cyaA::lacZY*	+	0.13	12,000	1.1×10^{-5}
E. coli	*cyaB::lacZY*	–	0.2	30,000	0.6×10^{-5}
E. coli	*cyaB::lacZY*	+	0.17	25,000	0.7×10^{-5}
BP 18323 Mod X	*cyaA::lacZY*	–	2,400	420	5.7
BP 18323 Mod C	*cyaA::lacZY*	–	150	ND	

[a] Bacteria were grown as described in text. Different rates of enzyme synthesis were determined during exponential growth. ND, Not determined.

Using an oligonucleotide complementary to the 5′ end of the *cyaB* as a primer for extension of the *cyaB* mRNA, we investigated whether the *cyaBDE* genes are expressed by readthrough or whether a second transcriptional start site exists in the *cyaA-cyaB* intergenic region, downstream from the transcription termination of the *cyaA* gene. Fig. 5 shows the primer extension products found, using as templates RNA isolated from strain 18323 grown in Mod X (lane 1) or Mod C (lane 2) conditions or RNA from strain BP347 (lane 3). In all the strains tested a discrete product was observed, corresponding to a transcriptional start site 30 bp upstream from the *cyaB* ATG initiation codon at a T residue. Transcription from this site occurs independently of a functional *bvg* locus and irrespective of modulative growth conditions. This second promoter, which activates transcription of the transport genes, has been shown to be at least five times weaker than the virulence-dependent *cyaA* promoter (12). These results demonstrate that expression of CyaA protein is positively regulated by the Bvg protein(s); the transport genes show a lower level of constitutive expression which is independent of virulence control.

To further study the regulation of the *cya* operon of *B. pertussis*, we constructed *cya-lac* fusions in *E. coli*. To construct the *cyaA::lacZY* fusion, a 1-kb DNA fragment was inserted into plasmid pNM482 (gift from P. Stragier), a ß-galactosidase fusion vector (16). This fragment extended from the *Pvu*II site 685 bp upstream of the *cyaA* translational start codon ATG to the *Sal*I site 330 bp downstream of ATG. The *cyaB::lacZY* fusion in pNM482 contained a 1.3-kb DNA fragment extending from the *Acc*I site located in the 3′ end of *cyaA*, 761 bp upstream of the *cyaB* translational start codon ATG, to the *Pst*I site 500 bp into the *cyaB* open reading frame. The *E. coli* Δ *lac* strain NM554 (20) was employed as the host for the plasmid constructions.

When plasmids containing either the *cyaA::lacZY* fusion or the *cyaB::lacZY* fusion were transformed into strain NM554, virtually no ß-galactosidase activity could be detected (Table 4). The obvious explanation for this lack of ß-galactosidase synthesis is that (i) the *cyaA* promoter requires *bvg* for expression, and (ii) the transcription initiation site 5′ to the *cyaB* gene is not recognized in *E. coli*. Therefore, a plasmid containing the entire *bvg* operon, present on plasmid pLA 5.7 (generously provided by R. Rappuoli) was introduced in trans to the *cyaA::lacZY* or the *cyaB::lacZY* fusions in strain NM554. As Table 4 shows, the expression of *cyaA* or *cyaB* was not affected by *bvg*. To check the functionality of the *cyaA::lacZY* fusion, we returned it to the chromosome of *B. pertussis* strain 18323 by homologous recombination. The amount of ß-galactosidase produced in the resulting strain grown in Mod X corresponded to the value expected from the known strength of *cyaA* promoter. In Mod C strains, ß-galactosidase synthesis was severely reduced (Table 4), confirming that the *cyaA::lacZY* fusion was functional. These results show that *bvg* has no effect on the *cya* promoter in *E. coli*. Thus, in reconstructed *bvg*-dependent regulatory systems in *E. coli*, although the transcriptional and environmental regulation of *fha*B is *bvg*-dependent, the expression of *ptx* and *cya* operons is not affected by *bvg* (14, 22; S. Goyard and A. Ullmann, FEMS Microbiol. Lett., in press).

DISCUSSION

AC from *B. pertussis* has been the subject of intense study for the past 15 years. At present, it is well established that the structural gene, cyaA encodes an approximately 200-kDa bifunctional protein endowed with both AC and hemolytic activities. The molecular features of the CaM-activated AC have been identified by limited proteolysis (9), substrate- and CaM-binding (11), and site-directed mutagenesis (2). These studies led to the identification of residues essential for catalysis and CaM binding and provided evidence for a two-domain structure of the N-terminal 400 residues long catalytic moiety.

Hewlett et al. (7) and Rogel et al. (22) have shown that the toxic form of AC is associated with the 216-kDa form of the cell-associated CyaA protein. We showed that the secreted, high-molecular-weight form CyaA protein also is endowed with toxin activity. The exact physiological role of the hemolytic activity has not yet been elucidated; our results suggest, however, that the main function of the hemolytic moiety of the CyaA protein is to channel the N-terminal catalytic domain across the bacterial envelope and into eukaryotic target cells. The residues responsible for the three functions — secretion, pore formation, and entry into target cells — have not yet been identified. The in-phase deletions constructed in the 3′ region of the *cyaA* gene did not alter the secretion of the truncated CyaA proteins, indicating that these residues, in regions highly conserved between *B. pertussis* CyaA protein and *E. coli* hemolysin, are not essential for secretion. The hemolytic activity of the truncated CyaA proteins was severely reduced and their toxin activity abolished. The requirement for structural integrity of the 200-kDa CyaA protein therefore appears to be more stringent for toxin activity than for hemolytic activity. These results suggest that distinct structural determinants within the CyaA protein are involved in secretion, pore formation, and entry into target cells. Further studies are needed to assess the possible role of each structural determinant in the different functions of the CyaA protein.

B. pertussis virulence-associated genes are positively regulated in a coordinate fashion by the *bvg* locus, which modulates their expression in response to environmental signals (13). The determination of the transcriptional organization of the *cya* operon revealed two distinct transcription start sites: (i) the *cyaA* gene is activated by a strong *bvg*-dependent promoter, and (ii) the *cyaBDE* transport genes are activated by a much weaker promoter which is not dependent on the *bvg* locus. The control sequences involved in *bvg* regulation are at present poorly understood. Furthermore, although both *ptx* and *fhaB* require the *bvg* locus for expression, the regulatory mechanisms involved are different (22).

The construction of *cya-lac* fusions provided the important information that the *cya* operon is not expressed in *E. coli* or activated by *bvg*. In this respect, *cya* appears to be similar to *ptx;* however, no prominent sequence similarities could be detected between the control regions of *cya* and *ptx* (5). The activation of *cya* and *ptx* by *bvg* therefore would be indirect, either by additional promoter-specific factors or by alternative σ factor(s) which could recognize DNA secondary structures upstream from the structural genes to activate transcription. The hypothesis of *Bordetella*-specific post-transcriptional modification(s) of the BvgA protein which would allow the formation of more efficient open promoter complexes cannot be excluded, however. For the moment, no generalization seems to apply to transcriptional activation of virulence-associated factors: each case might be unique.

ACKNOWLEDGMENTS

Financial support came from the Centre National de la Recherche Scientifique (URA 1129), the Ministère de la Recherche et de la Technologie (grant 88T0823), the Fondation pour la Recherche Médicale, and a grant of the Pasteur-Weizmann Council.

We thank S. Falkow, P. Stragier, and R. Rappuoli for the gift of plasmids; S. Wain-Hobson for critical reading of the manuscript; and J. Lortholary for secretarial help.

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DISCUSSION

DR. THOMAS: (Question inaudible).

DR. ULLMANN: We have not looked yet but you are perfectly right because one of the hypotheses would be that something like sigma 54 would be involved in the regulation of this protein by *bvg* but we have not looked for other factors.

DR. LOCHT: (Question inaudible).

DR. ULLMANN: We did not check for that. There might be. For instance, the mutants in E do not secrete cyclase at all but we did not look and that is a very interesting point since the secretion genes are expressed constitutively, maybe they serve for secreting something else too. I do not know what, maybe a protease which processes cyclase or something like that.

DR. RELMAN: For the record, I just want to say that none of the *vir*-activated fusions that we worked on are activated or repressed in *E. coli* with *bvg* although we do see some expressions. They are not being expressed at all (inadible).

DR. ULLMANN: The problem, you know, is that these β-galactosidase levels were measured for four days in order to get some yellow color so it is really a lot, it is very low.

A Strategy for Studying Pathogenic Mechanisms: Transposon Insertion Mutations with Conditional Phenotypes

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Much of our current understanding of *Bordetella pertussis* has resulted from the use of transposon insertion mutations. Transposons facilitate the cloning of virulence genes and have generally provided insight into the genetic basis of this bacterium's ability to produce and regulate a variety of virulence-associated gene products. Mutagenesis is carried out using conjugal suicide vectors, and the transposon's drug resistance confers positive selectivity to the insertion mutations. This protocol is amenable to bacteria such as *Bordetella* which do not have well-developed genetic systems, and the approach has been applied to many genera (1, 2).

B. pertussis virulence-associated genes that encode filamentous hemagglutinin, pertussis toxin, and adenylate cyclase-hemolysin (AC-Hly) were first identified by null mutations made with transposon Tn*5* (18). Expression of these loci requires a positive regulatory function, also defined by Tn*5* insertions, that is encoded by a region of the chromosome called *vir* (now also called *bvg*). Subsequently, Tn*5* derivatives constructed for making protein or transcriptional fusions were used to identify genes encoding surface-exposed and secreted proteins or genes which are trans-criptionally active (12, 19). The resultant mutations further expanded our understanding of *vir* functions by defining the role of *vir* in antigenic modulation and identifying loci which were either positively or negatively regulated by *vir*.

Another derivative of Tn*5* called Tn*5tac*1, generates conditional phenotypes by regulating transcription of target sequences adjacent to the site of transposon insertion (5, 6). Tn*5tac*1 and Tn*5* inserts are polar: in a region of transcribed DNA, they interrupt transcription at the point of insertion. However, transcription of sequences adjacent to one end of Tn*5tac*1 can be elicited from a strong outward facing promoter, P_{tac}. Transcription from P_{tac} is repressed by the transposon's *lacI* gene-product unless an inducer such as isopropyl ß-D-thiogalactoside (IPTG) is present. Thus, one class of Tn5tac1-generated insertion mutations have conditional phenotypes that are regulated by IPTG.

This paper reports on the isolation of Tn*5tac*1 insertion mutations in *B. pertussis* that express AC-Hly only in the presence of IPTG. AC activity in these strains responds to IPTG in a rapid and dose-dependent fashion and the analysis of one mutant extends our knowledge of AC-Hly regulation and secretion in *B. pertussis*. The implications of efficiently isolating conditional mutations that respond to exogenous regulation are discussed, because this technique should prove useful for elucidating bacterial pathogenic mechanisms.

MATERIALS AND METHODS

Bacteria and media. The phage, plasmids, and bacterial strains used are listed in Table 1. *B. pertussis* was grown in modified Stainer-Scholte medium (SSM) (11, 17) or on solid SSM supplemented with 10% fresh defibrinated sheep blood (Colorado Serum Company, Denver, Colo.) in an atmosphere of 95% air-5% CO_2 at 37°C. *Escherichia coli* was grown at 37°C on LN (complex) solid medium (14). Selective media contained one or more antibiotics: kanamycin (50 µg/ml), ampicillin (50 µg/ml), streptomycin sulfate (400 µg/ml), and nalidixic acid (50 µg/ml).

Mutagenesis with Tn*5tac*1. Construction of mobilizable suicide plasmids for Tn*5tac*1 mutagenesis of *B. pertussis* and other nonenteric bacteria has been described (6). Briefly, *E. coli* strain MC1061 harboring one of the parent plasmids (pMOJ3*oriT* or pSUP201) was

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* Presenting author.

TABLE 1. Bacterial phage, plasmids, and strains

Bacterial phage, plasmids, and strains	Relevant genotype	Reference
Phage		
λ::Tn*5tac*1	Oam29 Pam80	4
Plasmids		
pMOJ3*oriT*	amp^r, tet^r, *oriT*	[a]
pSUP201	amp^r, cam^r, Mob$^+$	17
pBC62	amp^r, *oriT*, tet::Tn*5tac*1	6
pBC63	amp^r, Mob$^+$, cam::Tn*5tac*1	6
pBR322	amp^r, ter^r	14
E. coli		
MC1061	Sup^o	2
S17-1	RP4-2-Tc::Mu-Km::Tn*7*	17
B. pertussis		
Tohama	phase I, virulent *B. pertussis*	[b]
BC19	nal^r derivative of Tohama I	6
BC23	str^r derivative of Tohama I	6
BC67	*cyaE*::Tn*5tac*1 (regulates AC-Hly)	6
BC68	*cyaE*::Tn*5tac*1 (regulates AC-Hly)	6

[a] Gift from E. Ward and W. Barnes, Department of Biological Chemistry and Molecular Biophysics, Washington University School of Medicine.

[b] A. Weiss, Department of Microbiology, Medical College of Virginia, Richmond, Va.

infected with a λ::Tn*5tac*1 phage (5) defective for replication and integration. MC1061 (Sup^o) will not support λ::Tn*5tac*1 propagation, and most of the *Kan*^{r} transductants result from transposition of Tn*5tac*1 (15). Tn*5tac*1 insertion into the tetracycline resistance gene of pMOJ3*oriT* produced pBC62; insertion into the chloramphenicol resistance gene of pSUP201 produced pBC63. Transformation of the Tn*5tac*1-containing plasmids into *E. coli* strain S17-1 yielded donor strains capable of mobilizing the suicide vectors by conjugation.

Donor *E. coli* strain S17-1 containing either pBC62 or pBC63 was mixed on SSM plates with recipient *B. pertussis* Tohama I strain BC19 (nal^r) or BC23 (str^r) in a donor:recipient ratio of approximately 1:10 and incubated at 37°C for 5 to 6 h. *Kan*^{r} exconjugants were identified by plating dilutions of the individual matings onto the appropriate selective media, screened for Hly ability on solid SSM containing sheep blood, and purified by single-colony isolation before further analysis.

Nucleic acids. *B. pertussis* chromosomal DNA was extracted as described previously (3), and *Sal*I fragments conferring *Kan*^{r} were cloned into pBR322 using standard techniques (14). Sequences of the cloned *B. pertussis* DNA were determined by the dideoxy method (20) using a GGAAACAGAATTC CCGGGGATCCCC primer to sequence from the O-end of Tn*5tac*1 (immediately 3′ to P_{tac}), AGTCATGCCCCGCGCCCACCGG to sequence from pBR322 (most 3′ distal to P_{tac}), TGTCAAACATGAGAATTCCTCCCG to sequence from the I-end of Tn*5tac*1, and GGGCTGCTTCCTAATCAGGAGTCG to sequence from pBR322.

AC assays. Mid- to late-log phase cultures (2 to 4 x 10^9 cells/ml) of *B. pertussis* grown in the presence of IPTG (0 to 500 μM) served as sources of AC activity. Whole cells were centrifuged and resuspended to A_{540} of 0.70 in phosphate-buffered saline (PBS) to provide intact cell-associated extracytoplasmic AC activity. Cells at the same concentration in 10 mM Tris-HCl (pH 8.0) with 150 mM NaCl were sonicated for 90 s in 30-s pulses to provide total cellular AC activity. For some experiments whole cells were incubated for 5 min at 37°C in PBS with 20 μg/ml trypsin (type XI from bovine pancreas, Sigma Chemical Co., St. Louis, Mo.) followed by 5 min at 37°C

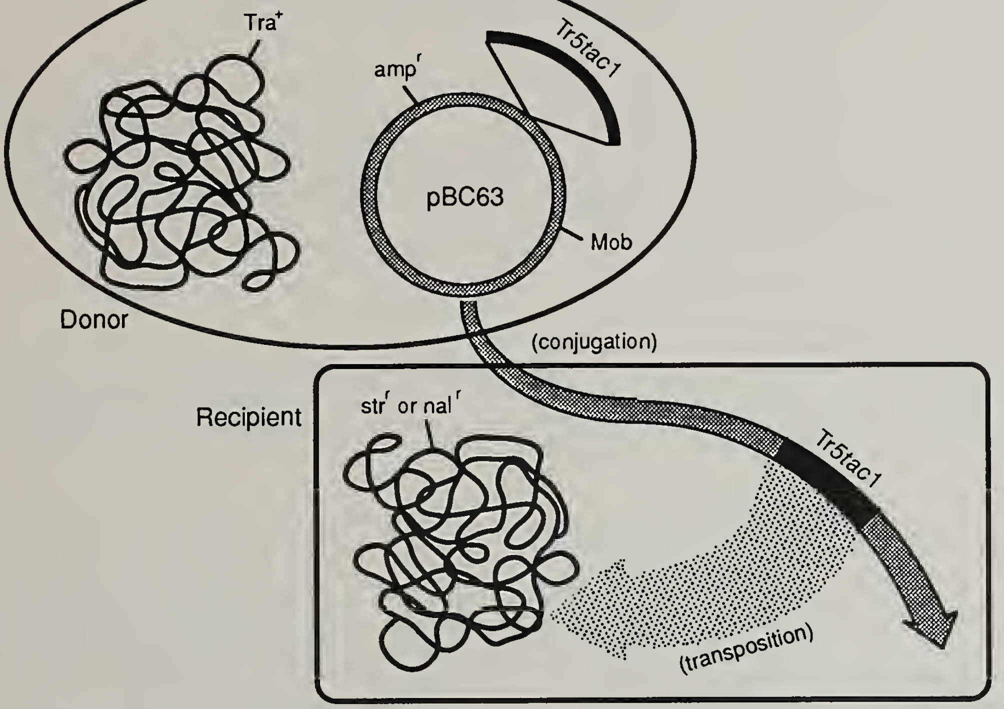

FIG. 1. Mutagenesis strategy employing transposon Tn*5tac*1. Donor *E. coli* strain S17-1 contains chromosomally encoded mobilization functions (Tra$^+$) that recognize the Mob site on pBC63 (and the *oriT* sequences of pBC62, not shown here) to mediate conjugative DNA transfer. Str^r or nal^r recipient *B. pertussis* becomes *Kan*^{r} as a result of Tn*5tac*1 transposition from the suicide plasmid into their chromosomes. Tn*5tac*1 was arbitrarily placed in the chloramphenicol resistance gene of pBC63 and the tetracycline resistance gene of pBC62.

in 20 µg/ml of trypsin-chymotrypsin inhibitor (Bowman-Birk inhibitor from soybean) to destroy extracytoplasmic AC activity. The trypsinized cells were washed by centrifugation, resuspended either in PBS or Tris-NaCl, and treated as described above.

AC activity was measured by the accumulation of cAMP in a mixture consisting of an enzyme source and a reaction cocktail containing calmodulin and ATP (6). Thirty µl of reaction cocktail was added to 30 µl of enzyme source and incubated at 30°C for 10 min. Reactions were stopped by adding 60 µl of 20% trifluoroacetic acid (TFA). We then centrifuged each sample for 4 min at 18,600 x g and concentrated the resulting supernatants to dryness in a rotary evaporator (Speed-Vac Concentrator, Savant Instruments, Inc., Farmingdale, N.Y.). All samples were stored at -70°C.

A description of the high-pressure liquid chromatography (HPLC) equipment (7) and conditions for the cAMP assay have been previously reported (6). To separate cAMP from other nucleotides, we developed a linear gradient from 100% solution A (0.015 M sodium acetate, 0.05% triethylamine adjusted to pH 4.0 with glacial acetic acid) to 85% solution A and 15% solution B (75% acetonitrile vol/vol in water) over a period of 15 min at a flow rate of 1 ml/min on a 130 x 4.6 mm C_8 reversed-phase cartridge column maintained at 30°C. The cAMP was quantitated by peak integration (absorbance at 254 nm) with reference to known standards.

Analytical methods. Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) using bovine serum albumin as a standard.

RESULTS

Isolation of Tn*5tac*1 insertion mutations. To isolate Tn*5tac*1 insertion mutations of chromosomal genes in situ, the transposon was delivered into *B. pertussis* on broad host-range suicide plasmids by conjugation with *E. coli* donor strain S17-1 (Fig. 1). Kanamycin-resistant exconjugants usually result from transposition of Tn*5tac*1 into the chromosomes of the recipient cells, because these donor plasmids cannot replicate in *B.*

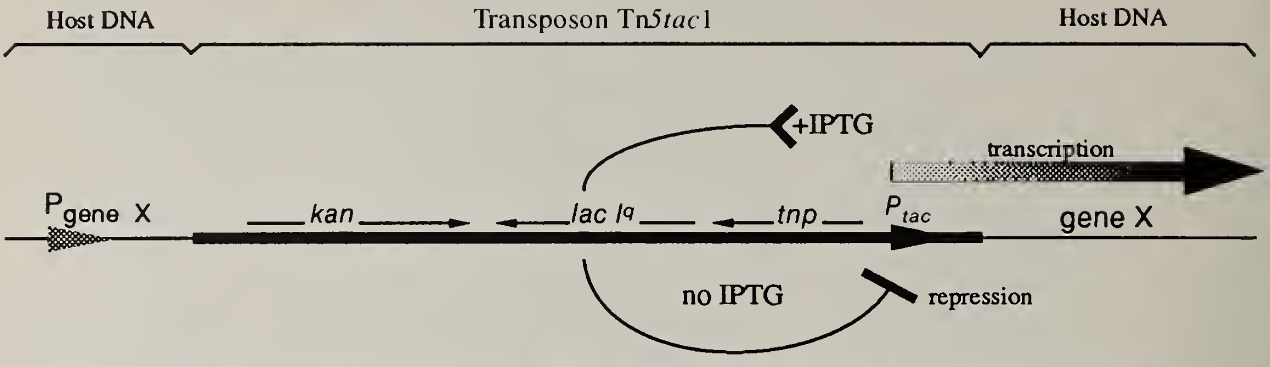

FIG. 2. Formation of a conditional phenotype using Tn*5tac*1. Important functional features of transposon Tn*5tac*1: *kan*^{r} gene; *lacI*^{Q}, *lac* repressor gene; and *tnp*, transposase gene. Because of the transposon's polarity, insertion of Tn*5tac*1 into the *gene X* target site of the recipient chromosome interrupts the gene's normal promoter function. In the presence of IPTG, *lacI* repression is alleviated and transcription of adjacent *gene X* sequences starts at P_{tac}. In the absence of IPTG, *lacI* repressor blocks transcription from P_{tac}. Thus, expression of X is subject to exogenous control by IPTG.

pertussis or other nonenteric gram-negative bacteria (16). We found *Kan*^{r} exconjugants at a frequency of 10^{-3} per donor cell using str^r as the contraselected marker in the recipient, compared to 10^{-6} per donor cell using nal^r (6).

The essential features of Tn*5tac*1 that permit DNA sequences to be placed under the transcriptional control of the transposon's P_{tac} promoter are diagrammed in Fig. 2. A nonhemolytic phenotype resulting from interruption of sequences required for hemolytic ability was chosen as a convenient screen for transposition of Tn*5tac*1 into the *B. pertussis* chromosome. About 1% of the *Kan*^{r} exconjugants were nonhemolytic, and eight independent nonhemolytic mutants were isolated. Two mutants, BC67 and BC68, became hemolytic when grown in the presence of IPTG. This indicates that Tn*5tac*1 can be used to isolate insertion mutations of chromosomal genes in situ and to produce mutant strains of *B. pertussis* with conditional, IPTG-dependent phenotypes.

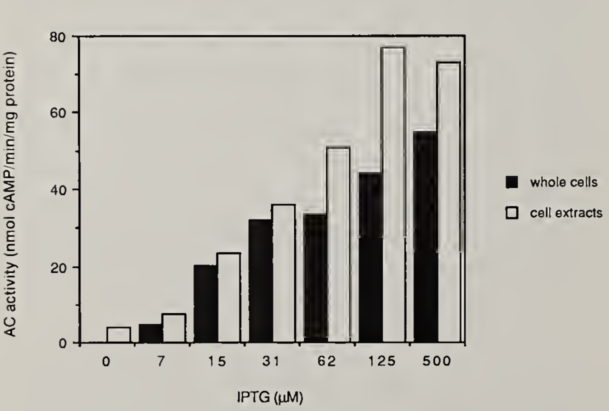

FIG. 3. Dose response of AC activity to IPTG concentration. *B. pertussis* strain BC67 was grown in various IPTG concentrations and the AC activity of whole cells (extracytoplasmic activity) and cell extracts (total cellular activity) was determined as described in the text.

Response of P_{tac}-mediated gene expression to IPTG. The hemolytic ability of *B. pertussis* is conferred by the bifunctional AC-Hly protein encoded by *cyaA* (8). Using an HPLC-based assay for measuring cAMP, the reaction product of AC acting upon substrate ATP, we showed that the AC activity and hemolytic ability of strain BC67 were coordinately regulated by IPTG (6). Most of the AC activity of the intact, wild-type bacteria is extracytoplasmic (but cell-associated); the remaining activity is present intracellularly (11). Fig. 3 shows that in strain BC67 the AC activity of whole cells (extracytoplasmic activity) and cell extracts (extra- plus intracytoplasmic activity) responded to exogenous IPTG in a dose-dependent fashion. Extracellular AC activity was undetectable in the absence of IPTG and induced at least 500-fold by 125 μM IPTG. At maximal induction (125 μM or greater), enzyme activity is equivalent to that of wild-type *B. pertussis* (6). These data imply that (i) P_{tac} serves as a promoter for *B. pertussis* RNA polymerase; (ii) the *lacI* gene product encoded by Tn*5tac*1 can repress transcription from P_{tac} in *B. pertussis*; and (iii) IPTG can enter this organism to relieve repression at P_{tac} and elicit dose-dependent expression of adjacent genes.

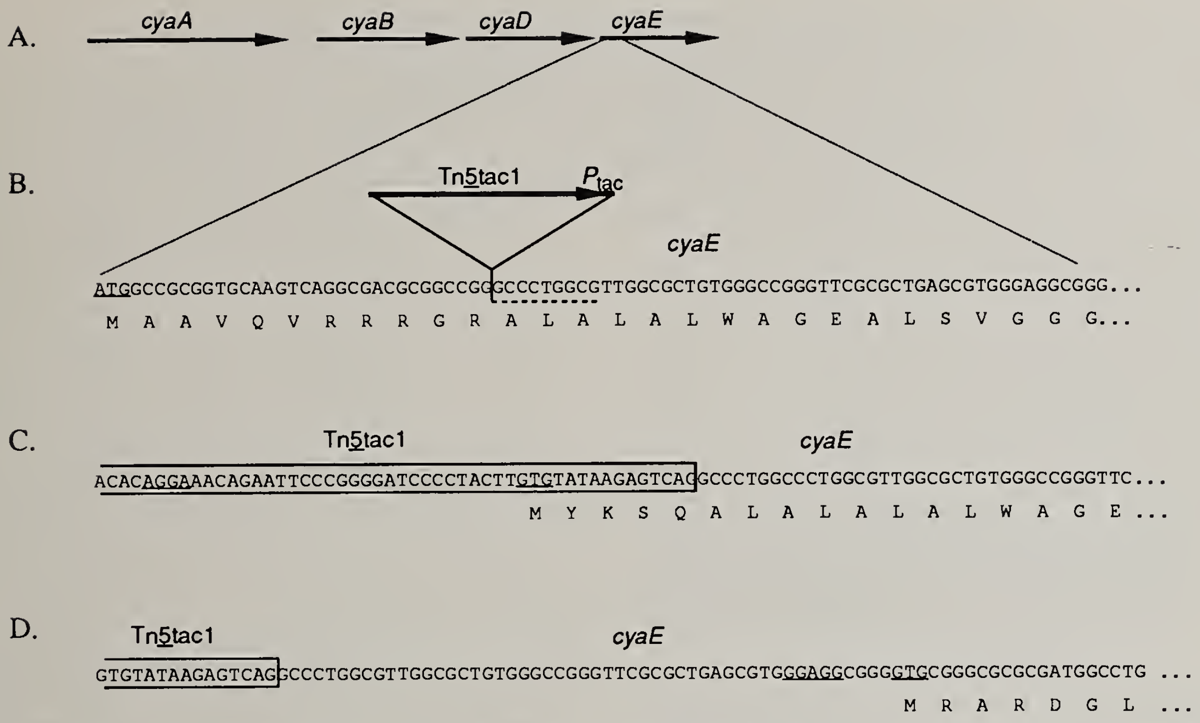
A.
cyaA
cyaB
cyaD
cyaE
B.
Tn5tac1
Ptac
cyaE
ATGGCCGCGGTGCAAGTCAGGCGACGCGGCCGGGCCCTGGCGTTGGCGCTGTGGGCCGGGTTCGCGCTGAGCGTGGGAGGCGGG...
M A A V Q V R R R G R A L A L A L W A G E A L S V G G G...
C.
Tn5tac1
cyaE
ACACAGGAAACAGAATTCCCGGGGATCCCCTACTTGTGTATAAGAGTCAGGCCCTGGCCCTGGCGTTGGCGCTGTGGGCCGGGTTC...
M Y K S Q A L A L A L A L W A G E ...
D.
Tn5tac1
cyaE
GTGTATAAGAGTCAGGCCCTGGCGTTGGCGCTGTGGGCCGGGTTCGCGCTGAGCGTGGGAGGCGGGGTGCGGGCGCGCGATGGCCTG ...
M R A R D G L ...

FIG. 4. Insertion site of Tn*5tac*1 in *B. pertussis* strain BC67. (A) Genes of the *cyaABDE* operon required for synthesis and secretion of AC-Hly (CyaA protein); *cyaBD* genes are required for CyaA secretion in *B. pertussis*, and they are homologous to the *hlyBD* genes of *E. coli* required for Hly export in that organism; *cyaE* gene is required for CyaA secretion and does not have a homolog in the *E. coli* hemolysin system. (B) DNA sequence of the first 84 nucleotides of *cyaE* and its deduced amino acid sequence (below). Tn*5tac*1 insertion site and transposon orientation in strain BC67 (*cyaE*::Tn*5tac*1) are shown. The start site of wild-type *B. pertussis cyaE* translation is underlined, and the nine-base-pair repeat typical of insertions of Tn*5* and its derivatives (2) is indicated by the dashed line under the DNA sequence. (C, D) Partial DNA sequence of transposon Tn*5tac*1 and adjacent *cyaE* in strain BC67. The potential ribosome-binding sites and initiation codons in the transposon and *cyaE* are underlined, and the deduced amino acid sequences from each are indicated below.

Mapping Tn*5tac*1 insertion of strain BC67. Although the *cyaA* gene encodes the complete AC-Hly protein (8), at least three other linked genes, *cyaB*, *cyaD*, and *cyaE* (Fig. 4), are needed for extracellular expression of CyaA (9). In addition, *cyaABDE* is subject to global control by the Bvg products of the *vir* region (13) which may act at *cyaABDE* through intermediate regulators. Thus, coordinate induction of AC and hemolytic activity by IPTG could result from a single Tn*5tac*1 insert near any of several genes in strain BC67. To differentiate among the possibilities, chromosomal DNA fragments from BC67 containing the entire Tn*5tac*1 transposon plus neighboring DNA sequences were cloned into pBR322 using the transposon's *Kan*^{r} as a selectable marker. The sequences adjacent to the Tn*5tac*1 insertion were determined using oligonucleotide primers for the ends of Tn*5tac*1. This showed that the Tn*5tac*1 insertion mutation was in the 5′ end of *cyaE*, and the transposon was oriented so as to direct transcription of *cyaE* in the presence of IPTG (Fig. 4B). In addition, the absence of AC activity and hemolytic ability in strain BC67 when grown at 25°C or in Vir$^-$ isolates of BC67 (independent of IPTG presence or absence) indicates that the upstream *cyaABD* remained intact and normally regulated (data not shown). Tn*5tac*1 was inserted between the 11th and 12th codon of the *cyaE* sequence, in a region encoding a stretch of hy-drophobic and basic amino acids resembling a typical bacterial signal sequence. Inspection of the sequence at the insertion site suggests that *cyaE* translation might start in Tn*5tac*1, resulting in a fusion protein where 5 amino acids fortuitously encoded by transposon sequences replace the first 11 amino acids of CyaE (Fig. 4C). Alternatively, translation could start at the 29th codon in *cyaE* (Fig. 4D). In either case, these experiments suggest that *cyaE* expression is necessary for extracytoplasmic AC activity and that the first 11 amino acids are not essential to CyaE function.

TABLE 2. Localization of AC activity during *cyaE*::Tn*5tac*1 induction with IPTG

Time (h)[a]	AC activity (pmol cAMP/min/mg) protein)			
	Extracytoplasmic[b]	Trypsin-resistant extracytoplasmic[c]	Intracellular[d]	Total cellular[e]
0	<1	<1	7,363	7,275
1	<1	<1	7,418	7,449
2	1,491	<1	5,931	7,530
3	4,236	<1	10,366	14,589
4	12,108	<1	17,448	26,374
6	19,631	<1	10,023	30,924

[a] Time after addition of 500-μM IPTG to culture medium of *B. pertussis* strain BC67.
[b] Whole cells assayed for AC activity as described in text.
[c] Whole cells treated with trypsin and assayed as above.
[d] Whole cells treated with trypsin, sonicated, and assayed as above.
[e] Cell extracts assayed as above.

The role of cyaE in the biogenesis of cyaA activity. Strain BC67 expresses very little AC activity in the absence of IPTG (Fig. 3). At maximal induction (125 μM IPTG), the activity of whole cells (a measure of extracytoplasmic AC) accounts for only 57% of total cellular AC activity (determined from cell extracts). Therefore, the remaining 43% may represent either (i) extracytop-lasmic AC activity that is unmeasurable in intact cells or (ii) intracellular activity of intact cells induced by IPTG. The former explanation is consistent with induction of *cyaE*-specified secretory functions by IPTG; the latter implies that transcription controlled by Tn*5tac*1 is responsible for increased activity as well as secretion of CyaA (6). To consider the role of *cyaE* in the biogenesis of AC activity, we examined intracellular and extracytoplasmic activity separately during induction of *cyaE* transcription.

Trypsin digestion of intact cells destroys the extracytoplasmic AC activity of *B. pertussis* (10). Sonication of these trypsin-treated cells (after inactivation of trypsin) liberates the intracellular cyclase activity protected from proteolytic inactivation. This protocol permitted us to determine the contribution of intracellular and extracytoplasmic activity to total cellular AC activity. Relying on IPTG's ability to titrate *lacI* repressor and rapidly induce transcription from Tn*5tac*1, the researchers added 500 μM IPTG to an exponentially growing culture of *B. pertussis* strain BC67 which had previously been growing in the absence of IPTG. At hourly intervals after the IPTG stimulus, the intracellular and extracytoplasmic compartmentalization of AC activity was determined (Table 2). After 1 h of IPTG induction, all cell-associated AC activity remained intracellular. Extracytoplasmic activity was first detected at 2 h, but total cellular activity remained unchanged. This finding indicated redistribution of intracellular activity to an extracytoplasmic location, and confirmed previous work showing that CyaE is needed for CyaA secretion (9). At 3 and 4 h postinduction, the total cellular and extracytoplasmic activity continued to increase, but intracellular activity also increased almost 2.5 times above the baseline activity determined prior to induction. At all time points 100% of the extracytoplasmic activity was trypsin-sensitive, and total cellular AC activity was equal to intracellular activity plus extracytoplasmic activity. We established therefore that the difference in activity of cell extracts and whole cells in Fig. 3 was due to an increase in the intracytoplasmic activity rather than to an inability to detect some fraction of the extracellular activity. These data suggest that *cyaE*::Tn*5tac*1-regulated transcription not only restores secretory function to the cell but also increases CyaA activity. Whether this increased AC activity is a result of transcriptional, translational, or direct protein interactions remains to be determined.

DISCUSSION

Mutagenesis of *B. pertussis* with transposon Tn*5tac*1 allowed us to isolate mutations with conditional phenotypes (6). In particular, properly oriented inserts in the 5′ end of *cyaE* placed transcription of this gene under the control of Tn*5tac*1's P_{tac} promoter. *B. pertussis* strains harboring such a cyaE::Tn*5tac*1 mutation did not express hemolytic ability or extracytoplasmic AC activity in the absence of exogenous IPTG. Both activities were restored by adding IPTG to the growth medium. Export of AC responded rapidly to exogenous IPTG (within a generation time, as seen in Table 2), and IPTG-induced enzyme activity exhibited dose dependence (Fig. 3). These observations confirm that *cyaE* is essential for the secretion of CyaA, the bifunctional AC-Hly protein (9). In addition, by considering the cellular location of AC activity during the induction of *cyaE* transcription, we found that CyaE increases intracellular CyaA activity.

Insertion mutations causing conditional phenotypes can help provide insight into pathogenic mechanisms, because pathogen-host interactions occur at or beyond the surface of the microbe and involve a complex, well-regulated network of bacterial gene products. Defining previously unknown genes and gene functions will be facilitated by transposon mutagenesis of chromosomal loci in situ, as shown here. Also, insertions can be directed to particular chromosomal regions of interest by mutagenizing cloned genes in *E. coli* using λ::Tn*5tac*1 (5) as a delivery vehicle. Subsequently, strains with IPTG-dependent phenotypes can be constructed and tested by introducing the mutagenized gene into its native chromosome by homologous recombination. With the appropriate Tn*5tac*1 mutations, one can regulate gene expression exogenously and explore how one gene product influences other bacterial functions just as we have done with *cyaE* and adenylate cyclase. These and other studies are aided by the unique features of Tn*5tac*1 discussed above, by near random insertional mutagenesis, and by a selectable drug-resistance marker to facilitate traditional genetic mapping and recombinant DNA techniques. These features are particularly valuable for studying bacterial pathogens with genetic systems that are not well developed.

The well-known influences of temperature, pH, O_2, amino acids, iron, etc., on the expression of virulence determinants indicate the sensory abilities of infectious organisms to detect their favorite host environment. A continually emerging concept in microbiology is that infection is a developmental process in which microbial gene expression is regulated by interactions with the host. The host environment will change and pathogen gene expression may correspondingly be altered, as colonization evolves into infection and infection ultimately gives rise to a disease state. Exogenous regulation of virulence phenotypes would allow temporal dissection of these developmental processes: it could be determined, for example, which stage of a successful host-pathogen interaction requires IPTG-dependent expression of a particular gene. Tn*5tac*1 inserts could also be used to exert quantitative control of gene expression by altering the concentration of the IPTG stimulus. In addition, temporal control of gene expression is possible by simply adding IPTG to the culture medium of a strain previously grown in the absence of IPTG. This approach may be especially well suited to cell and organ culture models of infection in which the timing and amount of IPTG administration may reveal the direct pathological consequences of expressing a particular virulence determinant.

ACKNOWLEDGMENTS

This work has been supported by National Institutes of Health research grant AI22243 to W.E.G., a grant from the Lucille P. Markey Charitable Trust and GM-37138 to D.E.B., and National Institutes of Health training grants GM-07200 and AI-07172 to the Washington University School of Medicine.

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DISCUSSION

PARTICIPANT: (Question inaudible).

DR. COOKSON: No, the only way that I have considered protein is by enzymatic activity so the interpretations are restricted by those observations.

DR. KNIGHT: Have you done any experiments to eliminate the possibility that there may be some additional downstream (inaudible) by *bvg* that is also being regulated?

DR. COOKSON: That is a good question, and I think that there are. I have not mapped the size of transcript, if that is what you are asking. I guess directly that would be the nicest thing to do but the mapping of the operon by Glaser, et al., has defined quite nicely the region of DNA that is required to get expression of the adenylate cyclase activity in complementation of some of Alison Weiss' Tn*5* mutants. Their analysis also included sequence analysis of a downstream, almost an entire kilobase of DNA and in that region there is no start site or ribosome binding site other than cyaE that is consistent with it encoding another gene function, and furthermore, preliminary results when we did the sequence analysis of the fragment containing Tn*5tac* and we took that particular clone and expressed it in maxi-cells. Our preliminary data suggest that there is only a single protein that is induced by transcription of Tn*5tac*.

DR. KNIGHT: (Question inaudible).

DR. COOKSON: That is a good experiment, but it has not been done.

DR. ULLMANN: (Question inaudible) and at what level about, what is the expression?

DR. COOKSON: Thank you very much and I agree with your comments. I think that one of the nice things about having a mutant such as the one that I have discussed with you today is that it allows you to begin to ask those sorts of questions because you can exogenously regulate its activity and so that hopefully with this mutant at hand we will be able to more clearly define the role of cyaE in adenylate cyclase regulation and its functional activity.

Production of *Bordetella pertussis* Fimbriae: Sequences Involved in Regulation and Identification of an Accessory Gene

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Fimbriae are extracellular filamentous proteins, that are found on many bacterial species and play an important role in the colonization of host surfaces through their ability to bind to particular receptors (5). *Bordetella pertussis* produces two distinct fimbriae, which were first identified as agglutinogens. There is some controversy over which agglutinogen corresponds to which fimbria; this report adheres to the school of Ashworth et al. (2) which assumes that the two fimbriae correspond to agglutinogens (or serotypes) 2 and 3. (Others maintain, however, that the second fimbrial serotype does not correspond to agglutinogen 3, but to agglutinogen 6 [4].) The serotype 2 and 3 fimbriae are composed of subunits with molecular weights of 22,500 and 22,000, respectively (10, 31). In addition to the genes coding for the serotype 2 and 3 subunits (*fim2* and *fim3*), *B. pertussis* contains a third fimbrial subunit gene, designated *fimX* (19). All three genes have been cloned and their DNA sequence determined (13, 18, 21). The authors have found evidence that *fimX* is not expressed in *B. pertussis* (unpublished experiments). Interestingly, the *fim2* and *fim3* genes may also be phenotypically silent, since a single *B. pertussis* strain may change its fimbrial serotype in the course of an infection (22). Thus, fimbriae appear to be subject to phase variation in *B. pertussis.*

Most *B. pertussis* virulence-associated genes are repressed when the bacteria are cultured in the presence of 20 mM SO_4^-. Studies have shown that the response to SO_4^- and other environ-mental signals is mediated by the products of the *bvg* locus (16, 17, 26). Further, it has also been shown that expression of *fim* genes is also repressed in the presence of 20 mM SO_4^- and that a transposon insertion in the *bvg* locus abolishes fimbria production (R. Willems, A. Paul, H. G. J. van der Heide, A. ter Avest, and F. R. Mooi, EMBO J., in press). Thus, the *fim* genes are subject to two control mechanisms, phase variation and positive control by the *bvg* locus. This report presents a model showing how these two regulatory phenomena may be coupled at the molecular level. The authors identify a locus on the chromosome of *B. pertussis,* distinct from *fim2, fim3,* and *bvg,* which is required for the production of serotype 2 and 3 fimbriae.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Strains and plasmids used are listed in Table 1. Conditions for growth have been previously described (19).

DNA techniques. Unless stated otherwise, DNA manipulations were performed following the protocols of Maniatis et al. (14). DNA sequences were determined

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype	Source or reference
B. pertussis strains		
Wellcome 28	Serotype 2^+, 3^+	25
B131	Serotype 2^+, 3^+; PhoA$^+$ (*xxx*::Tn*phoA*)	This work
B176	Serotype 2^-, 3^-; PhoA$^+$ (*fimE*::Tn*phoA*)	This work
B536	Serotype 2^+, 3^-	24
Clinical isolates		
B11	Serotype 2^-, 3^+	19
B12	Serotype 2^-, 3^+	19
B17	Serotype 2^+, 3^-	19
B18	Serotype 2^+, 3^-	19
Plasmids		
pEMBL8	Amp	6

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* Presenting author.

using the dideoxynucleotide chain termination method (27), combined with pEMBL8, on double-stranded DNA with Sequenase (USB, Cleveland, Ohio) according to manufacturer instructions.

Tn*pho*A mutagenesis. Tn*pho*A (15) mutagenesis and the determination of the PhoA phenotype were performed as previously described (11).

Immunological techniques. Total cell lysates containing approximately 5 x 10^7 bacteria were analyzed by immunoblotting essentially as described by Van Embden et al. (30) using monoclonal antibodies raised against sodium dodecyl sulfate-denaturated serotype 2 or 3 fimbrial subunits.

Rabbit experiments. Rabbits were infected as described elsewhere (F. R. Mooi, H. G. J. van der Heide, H. C. Walvoort, H. Brunings, W. H. Jansen, P. A. M. Guinee, FEMS Microbiol. Lett., in press). Rabbits, weighing 1.5 kg, were infected intranasally with a suspension of 10^6 bacteria. Bacteria were recovered by collecting material from the nasopharynx. This material was directly inoculated onto Bordet-Gengou agar plates, supplemented with the required antibiotics. The amount of bacteria

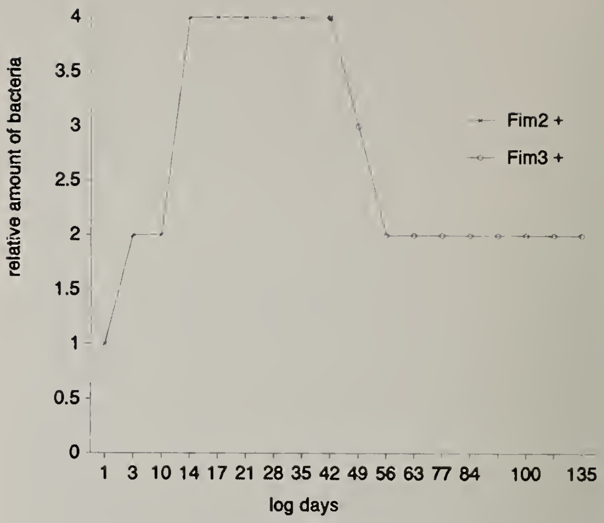

FIG. 1. Fimbrial phase variation of *B. pertussis* during infection of a rabbit. The rabbit was infected intranasally with the Tohama strain BP536 (Fim 2^+, Fim 3^+); at the indicated time points, the strain was re-isolated and inoculated on Bordet-Gengou plates. The serotype of the strains was determined by slide agglutination and immunoblotting.

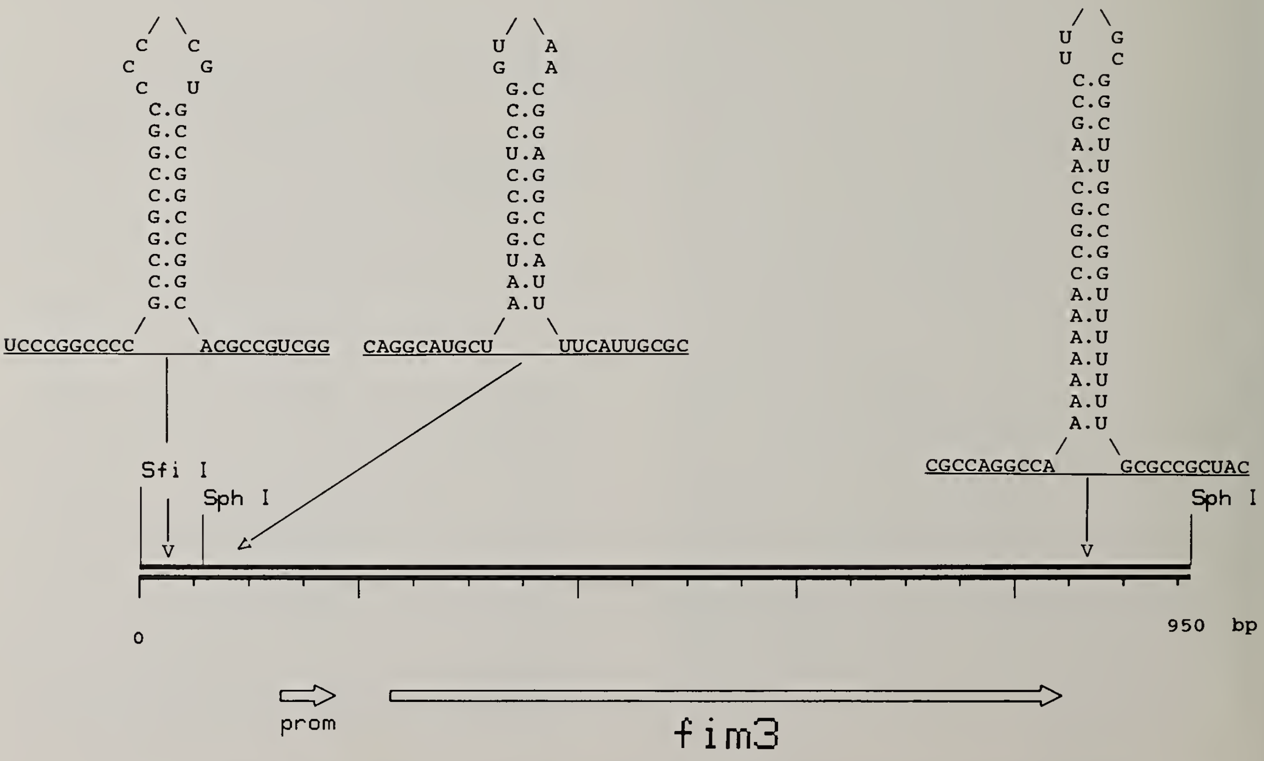

FIG. 2. Physical and genetic map of *B. pertussis* DNA harboring the *fim3* gene. Potential transcriptional terminators are depicted above the physical map. Prom, Promoter.

recovered was indicated as follows: 0, no colonies; 1, 1 to 50 colonies; 2, confluent growth in first streak; 3, confluent growth in first and second streaks; and 4, confluent growth in all four streaks. Colonies were restreaked and serotyped by slide agglutination.

RESULTS AND DISCUSSION

Fimbrial phase variation. As illustrated in Fig. 1, *B. pertussis* is able to cause a persistent infection in rabbits during which fimbrial phase variation can be observed. A rabbit was infected with the Tohama strain BP536 (serotype 2^+, 3^-), and, at different points in time, the strain was re-isolated from the nasopharynx and serotyped. It appeared that after approximately 40 days, the strain had changed its serotype from 2^+, 3^- to 2^-, 3^+, suggesting that the *fim2* gene had been switched off, and the *fim3* switched on. This assumption was confirmed by immunoblotting (not shown).

Fimbrial phase variation probably also occurs in infected humans, since clinical isolates may produce one or both fimbrial serotypes, although these strains contain copies of the *fim2* and *fim3* genes (19).

Location of the *fim* promoter. The *fim3* gene is enclosed by DNA sequences which may encode for (*rho*-independent) terminators (Fig. 2). This suggests that transcription is initiated close to the start of the gene. When the upstream regions of *fim2* and *fim3* are compared, significant homology is observed in a 40-base-pair (bp) region located approximately 40 bp upstream of the initiation codons (Fig. 3A), suggesting that the *fim* promoter is located in this region. A characteristic feature of the putative *fim* promoter region is a very long stretch of Cs. This stretch is followed by a sequence similar to the consensus sequence of -10 boxes (9). No typical -35 region is observed in the *fim* promoter, suggesting it is positively regulated (23); presumably the conserved region located upstream of the -10 box contains the binding site for the positive regulator. This region has been designated the activator binding region (AB) in Fig. 3.

The pertussis toxin (8), filamentous hemagglutinin (17), and adenylate cyclase genes (12) are also positively regulated by the *bvg* locus. In this light, it is significant that their promoter regions share similarities with the AB region of the *fim* promoter (Fig. 3B).

A

```
         -50       -40        -30       -20       -10
          :         :          :         :         :
     CTGTTTCCCACATCGGAATCAGCCCTCCCCCCCCCCCCTAAGACCTAAGATCGTGGC   fim2
     |   ||||||||     |||||||||||||||||||   |     ||   |
     CAAATTCCCACACAACCATCAGCCCTCCCCCCCCCC-GGACCTGATATTCTGATGCC   fim3
         |   AB region    |-.......22-23 bp......-|-10 |
```

B

```
     C...TTCCC-ACA.....ATCAGCCCTCCCCCCCCCC-..A.....TA...T..TG.C  fim cons
-55  CACCGTCCGGACCGTGC--TGA-CCCCCCTGCCATGGTGTGATCCGTAAAATAGGCAC  ptx
     ^    ^^^  ^^       ^ ^ ^^^ ^^  ^^             ^^   ^     *----->
-112 TGCAATCCA-ACACGG-CATGAACGCTCCT                              ptx
          ^^^  ^^^     ^^ ^ ^ ^^^^
-134 ATTCTTCCGTACATCCCGCTACTGCAATC                               ptx
         ^^^^  ^^^      ^    ^   ^
-156 ATTCGTCGT-ACAAAACCCTCGATTCTTC                               ptx
          ^^   ^^^      ^^    ^  ^
-160 AAATTTCCT-ACAAGTCTTGTATAAATAT                               fha
         ^^^^  ^^^        ^    ^
-550 GGCTTTTCTTACATGTTTC-CAGGATATG                               cya
         ^^ ^  ^^^       ^^^
```

FIG. 3. (A) Comparison of *fim2* and *fim3* promoter regions. Identical bases are indicated by (|). The activator-binding (AB) region and -10 box are indicated. (B) Comparison of the *fim* promoter consensus sequence (cons) with the promoter regions of a number of *bvg*-controlled operons. Capital letters indicate bases that are found in both the *fim2* and *fim3* sequences. Dots designate positions where the two *fim* sequences have different bases. Similarities between the *ptx* (8), *fha* (24), *cya* (7), and *fim* sequences are indicated by (^). Dashes indicate gaps introduced to increase the number of matches; -10 boxes are underlined. The transcriptional start in the *ptx* sequence is indicated by (*-->).

Sequences derived from *fim3* genes in the off phase contain a shorter C-track than sequences belonging to *fim3* genes in the on phase (Fig. 4). This finding strongly suggests that fimbrial phase variation in *B. pertussis* is caused by small mutations in the C-track located in the *fim* promoter region. These insertions and deletions probably arise by transient misalignment during replication (29). A similar mechanism has been found to regulate the activity of the *bvg* locus. However, in this case these mutations affect translation by introducing a frameshift (28).

Identification of an additional locus involved in fimbriae production. Generally five to eight polypeptides have been implicated in the phenotypic expression of fimbriae (5). Without exception, it has been found that the genes coding for these polypeptides are located in a single cluster. A different situation exists in *B. pertussis*, since there is no evidence that the *fim2* or *fim3* genes are part of a larger gene cluster. To identify accessory genes involved in the biogenesis of *B. pertussis* fimbriae, the authors mutagenized the Wellcome 28 strain with

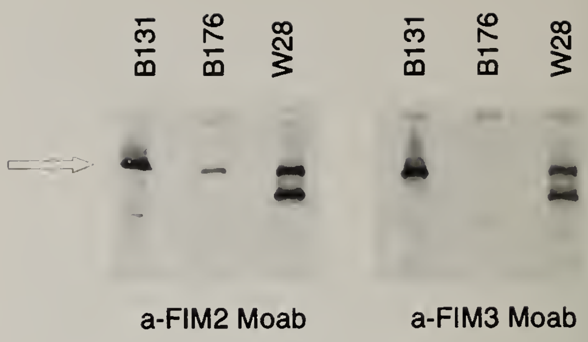

FIG. 5. Immunoblot illustrating the effect of the *fimE*::Tn*phoA* mutation on production of Fim 2 and 3 subunits. Strain B131 is a randomly picked Tn*phoA* mutant. Strain B176 carries the *fimE*::Tn*phoA* mutations. Both Tn*phoA* mutants were derived from the Wellcome 28 strain (W28). Blots were incubated with a monoclonal antibody directed against the Fim 2 subunit (a-FIM2 Moab) or the Fim 3 subunit (a-FIM3 Moab). The arrow indicates the position of the fimbrial subunit. Faster migrating bands represent degradation products.

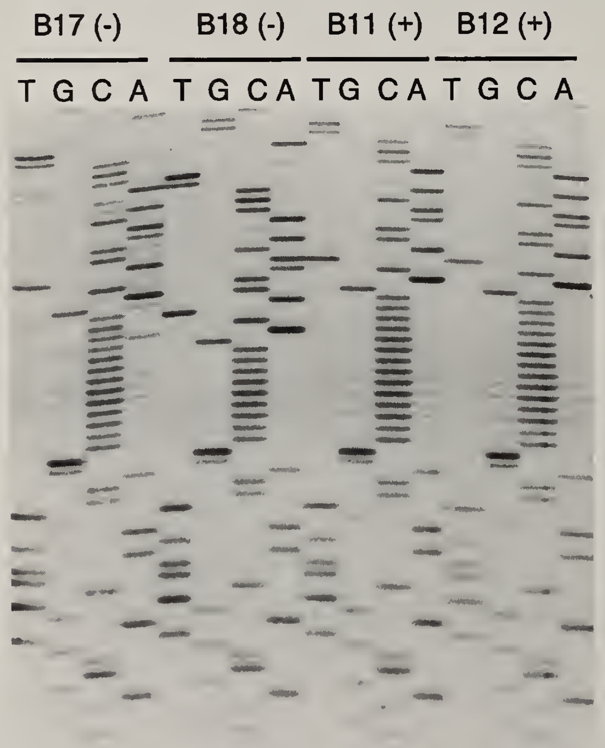

FIG. 4. DNA sequence of regions involved in phase variation. The *fim3* promoter sequences of four clinical isolates (B17, B18, B11, and B12) were determined. The sequences are identical except for the region containing the C-stretch. The phase of the *fim3* gene is indicated in parentheses.

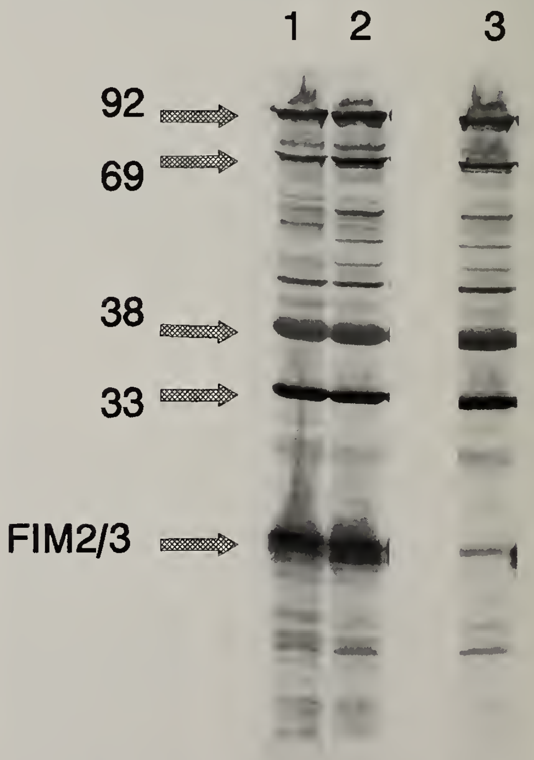

FIG. 6. Immunoblot of the W28 (lane 1), B131 (lane 2), and B176 (lane 3) strains. The blot was incubated with a mixture of monoclonal antibodies specific for the following outer membrane proteins: 92kDa, 69kDa, 38kDa, 33 kDa, and the Fim 2 and Fim 3 subunits. The position of these proteins is indicated on the left.

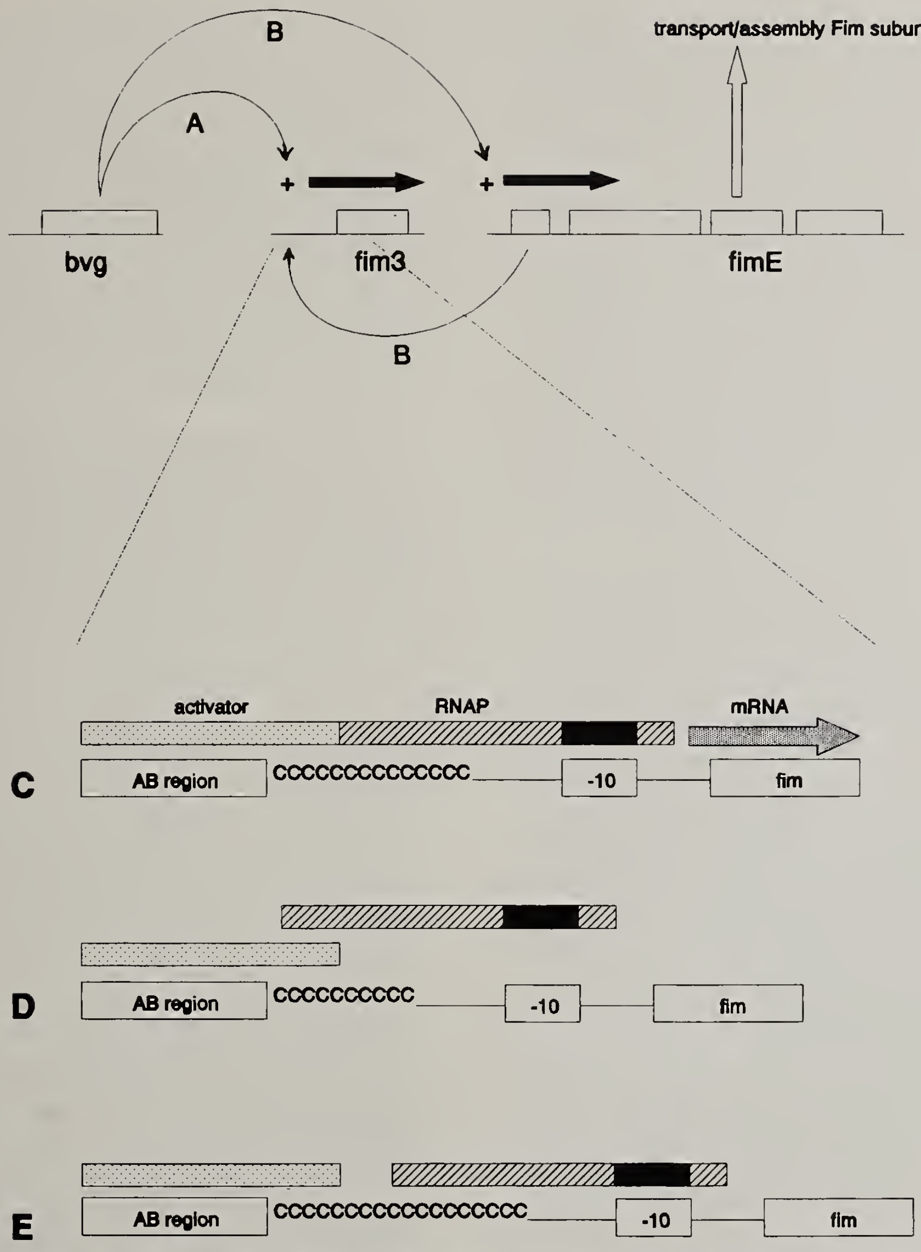

FIG. 7. A model for the regulation of the *B. pertussis* fimbrial genes. (A) An activator, encoded by the *bvg* locus, stimulates transcription of the *fim* gene directly by binding to the AB region and interacting with the RNA-polymerase molecule (RNAP), thereby enhancing initiation of transcription. (B) It is also possible that the *bvg*-encoded activator stimulates transcription of the *fim* gene indirectly by activating a second regulatory gene. In the model, the *fim* activator gene is presumed to be part of the fimbrial gene cluster harboring accessory genes. *FimE* codes for a periplasmic protein required for transport and assembly of fimbrial subunits (20). (C) Phase variation occurs by deletion (D) or insertion (E) of Cs in the C-stretch, which is located in the *fim* promoter region. Deletions in the C-stretch result in a partial overlap between the activator and RNAP-binding regions, causing mutual exclusion of these molecules. Insertion of Cs increases the distance between the activator and RNAP, thereby preventing contact.

Tn*pho*A, selected phoA$^+$ mutants, and analyzed these by means of immunoblotting. The Wellcome 28 strain produces both Fim 2 and 3 subunits; consequently, the authors reasoned that a Tn*pho*A mutant unable to produce both subunits most likely harbored an insertion mutation in an accessory fimbrial gene required for transport or assembly of fimbrial subunits. One Tn*pho*A mutant, designated B176, showed the desired phenotype (Fig. 5). In strain B176, low amounts of Fim 2 and 3 subunits are produced, and this strain does not agglutinate with serum directed against serotype 2 or 3 fimbriae (not shown). Such a pleiotropic effect on fimbria production could be caused by a transposon insertion in the *bvg* locus. Therefore, the authors determined whether the insertion mutation affected production of other *bvg*-controlled outer membrane proteins. No effect of the transposon insertion was found on the production of the 33-, 69-, and 92-kDa outer membrane proteins (Fig. 6.), all three of which are subject to *bvg* control (1, 3). The production of the 38-kDa outer membrane protein, which is not *bvg*-regulated, was also not affected in strain B176 (Fig. 6). Strain B176 therefore harbors Tn*pho*A in a locus distinct from *fim2*, *fim3*, and *bvg;* this locus has been designated *fimE*.

Since strain B176 has the PhoA+ phenotype, the transposon is located in a gene coding for an extracytoplasmic polypeptide. PhoA production appeared to be *bvg* dependent, indicating that *fimE* is part of the *bvg* regulon. Based on these observations, the insertion is presumably located in one of the accessory genes required for transport, assembly, and anchorage of fimbrial subunits. A likely candidate location is the gene coding for the periplasmic transport protein, since it has been shown that fimbrial subunits are degraded in the absence of this protein (20), thereby explaining the low amount of fimbrial subunits detected in strain B176.

In summary, the *fim* genes are subject to two control mechanisms which act at the transcriptional level: phase variation and positive regulation by the *bvg* locus. Furthermore, there is evidence for a locus, distinct from *fim* and *bvg*, that is probably required for a post-translational event in fimbria production. Fig. 7 presents a model incorporating these findings. This model hypothesizes that an activator stimulates transcription of the *fim* gene by binding to the activator-binding region and interacting with the RNA-polymerase molecule. Phase variation occurs by insertion or deletion of Cs in the C-stretch which is located between the activator-binding region and the -10 box. The distance between these two regions is 22 to 23 bp in active *fim* promoters. Deletions in the C-stretch decrease this distance and may cause a partial overlap between the activator- and RNA-polymerase-binding regions, resulting in a mutual exclusion of these molecules. It is not yet clear whether insertions which increase the distance between the two regions to more than 23 bp affect promoter activity. It is conceivable, however, that if this distance becomes too large, the activator may not be able to interact with the RNA-polymerase molecule. The activator may be encoded by the *bvg*A (26) gene: in that case, the *bvg* locus stimulates transcription of the *fim* gene directly. It is also possible that the *bvg* locus acts indirectly by activating a second regulatory gene. This second gene may be part of a gene cluster harboring accessory fimbrial genes such as *fimE*. The *fimE* product is required at a post-translational level for transport and/or assembly of fimbrial subunits, as has been proposed for the *faeE* product of the *Escherichia coli* K88 fimbrial gene cluster (20).

ACKNOWLEDGMENTS

Part of this work was supported by a North Atlantic Treaty Organization travel grant (0527/87). R. J. L. W. was supported by the Dutch Organization for Scientific Research, grant 900-514-106.

Monoclonal antibodies were generously provided by Jan Poolman and Betsy Kuipers.

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DISCUSSION

DR. SHAHIN: If you quantitate your bacterial recovery from the lungs instead of just sample percent of your animals colonized, do you see any differences?

DR. MOOI: You are asking me whether I looked at colony forming units in the lungs of mice?

DR. SHAHIN: Yes.

DR. MOOI: No, we might be going to do that but it is a matter of having the hands and the people. The way we do it is simple so that we can do a lot of experiments.

DR. SHAHIN: I understand that, but our experience with our mutants has been different in collaboration with Theresa Finn using Stefan Knapp's transposon insertion mutant that deletes toxin activity. We see a two-to-three-fold log reduction in bacterial persistence in the lung so that the bacteria are present but they are reduced. We have not looked in the trachea yet. The other thing that we have noticed is if he uses a wild type parental strain into which you inserted a strep resistance gene, this has been done with Rino Rappuoli, you see a reduction in the wild type strep resistance mutant compared to the parental wild type. So you have to be very careful in the background that you are putting your deletion mutations in because antibiotic resistance can affect bacterial persistence as well.

DR. STIBITZ: Have you looked in the Tohama III background at the rate of fimbrial phase variation? I ask this because with the vir locus we see a pronounced difference between the Tohama I and the Tohama III background which is also occurring by this frame shift mechanism.

DR. MOOI: No, we only worked in Tohama I strains.

DR. STIBITZ: Your fim minus or fim-III minus strain which has deleted, I believe it was five C-residues, do you see reversion of that? Can you see reversion of that in vivo?

DR. MOOI: That is an interesting question and of course to put it in broader perspective, it would be very interesting to know whether, for example, 30 years ago all these genes were switched on but then in the course of time they were more or less permanently inactivated. I think the chance that the fim-X gene will be switched on is very low because the mechanism by which these mutations occur and so insertions and deletions, is dependent on the length of the C-track and so the C-track becomes shorter and shorter then the chance of getting back the wild type situation will be very small.

DR. QUENTIN-MILLET: I found your presentation very interesting and I think there is something that is very different in *Bordetella* than in other pathogenic bacteria is that here we very seldom speak about antigenic variation whereas if you take any other type of bacteria we are talking about sero-group and sero-subtype. I think that perhaps in other pathogenic bacteria, this is what they have developed to sort of allude the immune system long enough to establish infection. Now, in pertussis maybe we have other tricks such as putting out all these extracellular proteins to allude the immune system, to establish infection, and I think you are suggesting that perhaps antigenic variation is taking place later in infection. Could you comment on what you believe the physiological purpose of this is as far as pertussis is concerned?

DR. MOOI: I understand that you are intrigued by the antigenic variation of *Bordetella*?

DR. QUENTIN-MILLET: No, actually the lack of it; the fact that there is very little antigenic variation. We are just talking about the fim, the antigenic variation in fimbriae and this does not seem to be of importance in very early colonization but perhaps later on. So what is the advantage as far as *Bordetella pertussis* is concerned to switch on this antigenic variation later on while infection seems to already be established? Do you have any thoughts?

DR. MOOI: Well, first of all, a number of people have suggested that *Bordetella pertussis* shows little antigenic variation because there are so many immunosuppressive toxins. But you can imagine that later in disease you will get an antibody response against particular antigens and especially fimbriae may be very effective. Not because some sort of function is blocked but because the antibodies against fimbriae are very well able to agglutinate the bacteria and that is one of the ways to prevent attachment, for example. So I think that this is maybe one explanation why fimbriae do show antigenic variation while the others do not.

Also, another point is that most of the virulence factors of *Bordetella* are loosely associated with the cell, and degraded or excreted. Fimbriae are very firmly attached to the cell. I think antibodies directed against an antigen which is excreted maybe will not neutralize the function of that toxin but it will not affect the cell itself too much, while in the case of fimbriae that is different.

DR. THOMAS: When you take out a lung, you have alveolae in the lung but you also have all the bronchials feeding that alveolae. So you cannot say when we isolate an organism either in terms of it being present or in terms of colony forming units from the lung, that it is an infection of non-ciliated alveolae. That may well represent infection of the bronchials feeding those alveolae as much as infection of the alveolae itself and so your implication that infection of trachea was infection of the ciliated epithelium and infection of lung was infection of non-ciliated epithelium is not necessarily a clear distinction.

DR. MOOI: I should probably be more specific and say the lung alveolae where I do not think you have very many ciliary cells.

DR. RAPPUOLI: I would just like to make one comment about one thing which we already brought up yesterday. In reference to the first part of your talk is the use of Alison Weiss' mutant, the pertussis toxin minus mutant. That mutant we know does not produce any acellular toxin but is overloaded with cell associated toxin so I think all those conclusions about toxin being involved in adhesion and things like that cannot be drawn. You cannot make a conclusion using that statement about the role of pertussis toxin. You need to have a PT-minus strain.

DR. MOOI: Alison Weiss made two pertussis toxin mutants and in one the toxin was available on the outside of the cell. That is not the one we took. We took the other one which I think was essentially tox minus.

DR. RAPPUOLI: Alison might comment on that. Both of them have a Tn*5* and S-3 so the toxin is not secreted but if you look at the toxin associated with cells, it is very high. It is higher than wild types strains.

DR. PITTMAN: I would like to ask the question in regard to the rabbit infection. You noted that it was a long duration and that has been reported in the literature and I have observed it. But I have not observed any effect of pertussis toxin in the rabbit. In my experience, they do not produce leukocytosis and they do not produce hypoglycemia in response to pertussis vaccine. Do you have any comments of the difference between the mouse and the rabbit in regard to the rabbit response to pertussis toxin?

DR. MOOI: We were also not able to find any leukocytosis in the rabbit and I think one of the problems is that it depends on the age of the animals. At least, that has been told to me and maybe the rabbits we used were too old. But I must admit that I am not too familiar with this field.

DR. PITTMAN: But it seems like the long duration in the rabbit is due to absence of antitoxin being produced. Is that true?

DR. MOOI: It is?

DR. PITTMAN: Do they produce antitoxin? They have no response to pertussis toxin during the infection. They also have treated with pertussis vaccine, they have a drop in normal histamine sensitivity.

DR. STIBITZ: Perhaps this can be clarified in private discussions.

Mutational Analysis of the Adenylate Cyclase Toxin of *Bordetella pertussis*

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One of the virulence factors of *Bordetella pertussis* is an adenylate cyclase (AC) toxin capable of causing a dramatic rise in cAMP levels in mammalian target cells as well as lysing red blood cells. The toxin itself is an AC enzyme that enters target cells where it catalyzes the conversion of intracellular ATP to cAMP. In phagocytic leukocytes, this massive accumulation of cAMP results in paralysis of killing function (3, 17). This toxin is believed to obstruct the host's immune response through such paralysis of immune effector cells. An important role is based on the fact that mutants lacking the AC toxin are avirulent in the infant mouse model of pertussis infection (23).

Recently this toxin has been purified to homogeneity by two groups who reported finding 216-kDa (11) and 200-kDa (18) invasive proteins, respectively. It has been demonstrated that the purified toxin was hemolytic (I. E. Ehrmann, V. Gordon, M. C. Gray, L. Gray and E. L. Hewlett, submitted for publication). This single molecule, therefore, has three measurable activities: (i) AC enzyme activity is measured in vitro by converting radioactively labeled ATP to cAMP, (ii) AC toxin activity is measured by the rise in cAMP in target J774 cells, and (iii) hemolytic activity is assayed on blood agar plates.

The gene encoding the AC toxin has been cloned and sequenced (8); it predicts a 1706 amino acid protein. Glaser et al. have demonstrated that the enzymatic domain is at the amino terminus (7, 8). Homology between the AC toxin-hemolysin (Hly) and the *Escherichia coli* Hly operon has been established (9). The structural gene *cyaA* for the AC toxin is homologous to the *E. coli* Hly structural gene *hlyA*. Genetic and biochemical evidence concur that a single gene produces a protein that is both toxic and hemolytic. Downstream from *cyaA*, three open reading frames have been identified. Genes *cyaB* and *cyaD* are homologous to the *E. coli* genes *hlyB* and *hlyD* which are responsible for secreting Hly in *E. coli* (5, 10, 20, 25). The third open reading frame, *cyaE*, has no homolog in the *E. coli* Hly operon (9). A *cyaC* gene has been identified upstream of *cyaA* (E. M. Barry, A. A. Weiss, I. E. Ehrmann, M. C. Gray, E. L. Hewlett, and M. S. Goodwin, submitted for publication) that is homologous to the *E. coli hlyC* gene (16). The C gene functions to activate the structural gene product post-translationally.

This research began genetic dissection of the AC toxin to further define its domain structure. Chromosomal mutations in the structural gene revealed regions necessary for toxin and Hly but not enzyme activity. Mutations in downstream sequences support a role for these genes in transporting the AC toxin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in these studies are listed in Table 1. *E. coli* strains were grown on L-agar plates (14) supplemented with antibiotics where indicated at the following concentrations: kanamycin, 50 µg/ml; ampicillin, 100 µg/ml; gentamicin, 10 µg/ml; and tetracycline, 15 µg/ml. *B. pertussis* strains were grown on Bordet-Gengou agar (BGA) (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 15% sheep's blood. Antibiotic concentrations for *B. pertussis* strains were as above or kanamycin, 25 µg/ml; nalidixic acid, 30 µg/ml; and streptomycin, 300 µg/ml. For urea extract preparations, *B. pertussis* strains were grown in Stainer-Scholte (SS) medium (GIBCO Laboratories, Grand Island, N.Y.). SS-Mg plates contained SS broth with 1.5% agar, supple-

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* Presenting author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source (reference)
B. pertussis		
BP338	Nal^r Tohama I	(24)
BP348	BP338 *cyaA*::Tn*5*	(24)
SM001	Sm^r derivative of BP338	This manuscript
BPD358	SM001 with 60 amino acid deletion in *cyaA*	This manuscript
BPD377	SM001 with 157 amino acid deletion in *cyaA*	This manuscript
BPK355	SM001 with truncated *cyaB* and *cyaD*	This manuscript
BPR327	SM001 *cyaE*::pEB317	This manuscript
Plasmids		
pRTP1	Vector for return of cloned sequences to *B. pertussis* chromosome	(19)
pSS1129	pRTP1 with a gentamycin resistance marker	(19)
pUC-4K	Vector with Kan^r cassette	Pharmacia, Inc., Piscataway, N.J.
pRK2013	Kan^r, Tra functions of RK2 cloned in ColE1	(6)
pUW956	Kan^s derivative of pRK2013	(24)

Abbreviations: Nal^r, naladixic acid resistant; Sm^s, streptomycin sensitive; Kan^r, kanamycin resistant; Kan^s, kanamycin sensitive.

mented with 0.15% bovine serum albumin and 20 mM $MgSO_4$.

DNA manipulations. Enzyme reactions were performed according to manufacturer specifications. Ligations were performed with fragments cut from Tris acetate buffered (0.16 M Tris base, 0.08 M sodium acetate, and 8 mM EDTA), low-melting-point agarose gels (FMC Corp., Rockland, Maine). Five µ of each fragment in agarose plus ligase were incubated overnight at room temperature. Ligation mixes were melted at 65°C and diluted 1:5 in water prior to transformation into *E. coli* HB101.

Conjugation and recombination. Plasmid pSS1129 is a derivative of pRTP1 with a gentamicin resistance gene. These plasmids were designed by Stibitz et al. to allow the exchange of cloned, mutated *B. pertussis* sequences with the homologous wild-type sequence in the chromosome (19). The researchers introduced pRTP1 or pSS1129 containing a mutated fragment (as described in Results) into a streptomycin-resistant strain of BP338, *B. pertussis* strain SM001, by conjugation with the helper plasmid pUW956 (21), a kanamycin-sensitive derivative of pRK2013 (6). Mating mixtures contained 800 µl of SM001 at an OD_{600} of 1.0 plus 100 µl of each of the *E. coli* strains at the same concentration. Matings were carried out on SS-Mg plates at 37°C. After 6 h, the bacteria were transferred to BGA plates with antibiotics to select for single recombination events where the entire plasmid had integrated into the chromosome. These colonies were then plated on streptomycin-containing BGA plates to select for double recombination events where the plasmid had been lost from the chromosome and the mutated sequence had replaced the wild-type chromosomal copy. Chromosomal mutations were confirmed by Southern blot hybridization (2) using positively charged nylon membranes.

Urea extracts. Urea extracts of *B. pertussis* strains were prepared as described previously (12).

AC enzyme assay. Following 24 h of growth on BGA plates, *B. pertussis* strains were resuspended in SS broth to an OD_{600} of 1.0. Whole-cell assays were conducted directly on these suspensions. For lysed cell assays, the bacteria, maintained in an ethanol ice bath, were sonicated for 4 min at 60% power in a Branson 450 sonifier. In vitro AC enzyme assays were performed as described previously (12) with 1 µM calmodulin.

AC toxin assay. AC toxin activity was assayed by measuring the increase in cAMP levels in target J774 cells as described previously (12).

Infant mouse model. Intranasal challenge of 6-day-old infant BALB/cByJ mice was performed as described previously (23).

RESULTS

Structural gene mutations. Included within the 216-kDa AC toxin molecule are domains responsible for enzyme activity as well as for toxin and Hly activity. The catalytic domain has previously been identified in the protein's amino terminus (8). Specifically, residue 58 has been demonstrated to be essential for the catalytic site (1, 7). To identify other functional domains, the researchers made mutations in the structural gene *cyaA* and assayed the resultant strains for loss of individual activities.

The chromosome of mutant BPD377 contains a 471-base-pair in-frame deletion in *cyaA*. This lesion was created by deleting a *Bgl*II fragment and results in the loss of amino acid residues 621 to 778. This deletion was accomplished by cloning the 2.5-kilobase *Bam*HI/*Eco*RI fragment of *cyaA* (Fig. 1) into vector pSS1129. The resulting plasmid pEB364 was digested with *Bgl*II. The digest was applied to a low-melting-point agarose gel where the large fragment was isolated and allowed to recircularize, thereby creating pEB377. *E. coli* harboring pEB377 was then mated with *B. pertussis* strain SM001, a streptomycin-resistant wild-type strain, to allow recombination between mutant and wild-type *cyaA* alleles. The first recombination event resulted in integration of the entire plasmid into the chromosome and was selected with gentamicin. These colonies were transferred to streptomycin-containing BGA to select for a second recombination event in which the mutated sequence replaced the wild-type chromosomal copy.

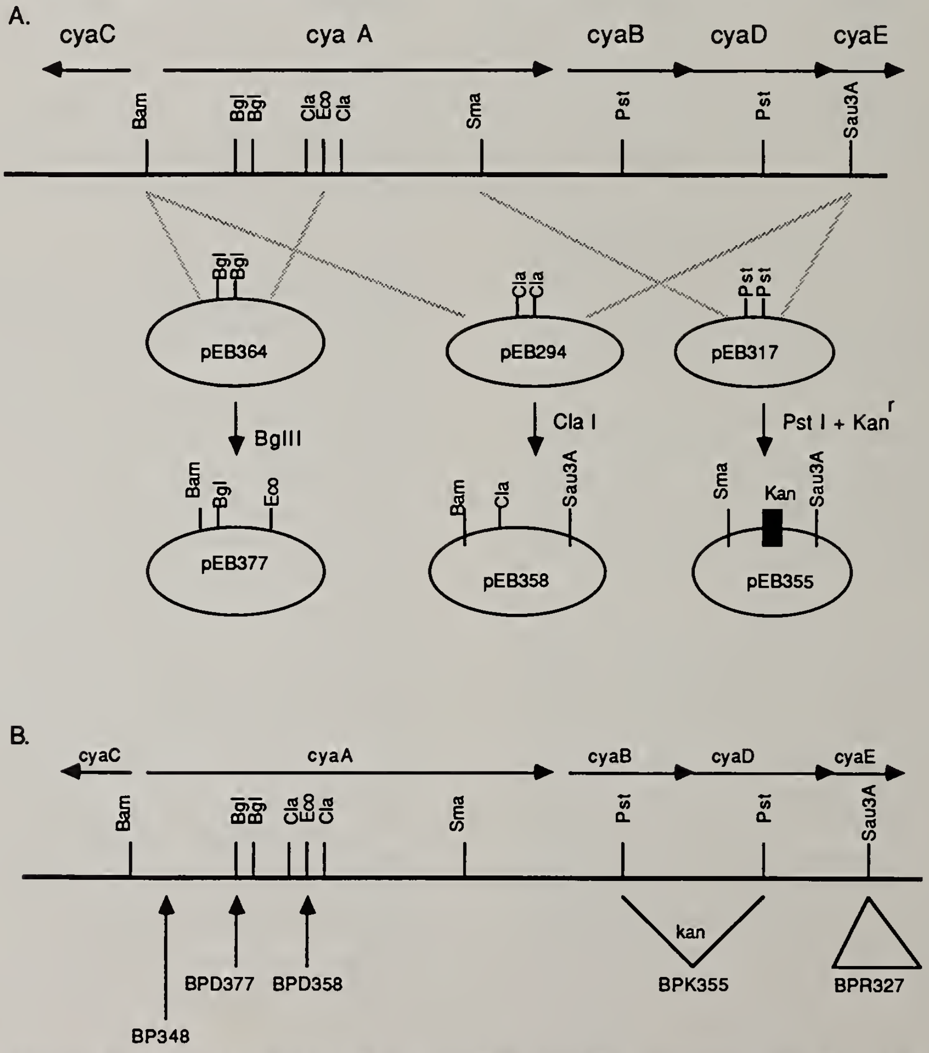

FIG. 1. Construction and location of mutations in the AC toxin genes. (A) Strategy for mutation of fragments cloned in pSS1129. (B) Chromosomal location of each mutation following its return to *B. pertussis*.

TABLE 2. AC and hemolytic activity in *cyaA* mutants

Strain	Hemolytic activity[a]	AC[b]	
		Enzyme	Toxin
SM001	+	+	+
BPD358	–	–	–
BPD377	–	–	–
BP348	–	–	–

[a] Hemolytic activity was measured on blood agar plates.

[b] Urea extract preparations were used in enzyme and toxin assays.

Transconjugants containing the deletion were identified as those that were nonhemolytic on blood agar plates. Verification of the deletion in the *Bordetella* chromosome was accomplished by Southern blot hybridization.

Mutant strain BPD358 contains a 180-base-pair *Cla*I deletion in *cyaA* (Fig. 1). This lesion results in the loss of amino acid residues 826 to 886. The deletion was created in the cloned 9.4-kilobase *Bam*HI/*Sau*3A fragment (pEB317). The lesion was returned to the SM001 chromosome as described above.

A Western blot of urea extracts of mutant strains BPD377 and BPD358 verified that the AC toxin protein was made and migrated faster than the wild-type toxin on sodium dodecyl sulfate-polyacrylamide gel electrophoresis appropriately for each deletion (data not shown).

These structural gene mutants were assayed for enzyme, toxin, and Hly activity (Table 2). Strain BP348, a previously characterized Tn*5* insertion mutant in *cyaA* which has no AC enzyme, toxin, or hemolytic activity, was included as a control (24). Both mutants were nonhemolytic on blood agar plates. Also, both mutants had no toxin activity; they could not cause a rise in cAMP levels in target J774 cells. These data indicate that the domains necessary for hemolytic and toxin activity were either deleted or disrupted by the deletions. Both mutants retained enzymatic activity. This result concurs with previous data assigning the catalytic domain to the amino terminus of the protein (10). The catalytic domain remained intact with these downstream mutations.

Downstream mutations. Glaser et al. have identified three open reading frames downstream from *cyaA* by sequence analysis (9). The first two, *cyaB* and *cyaD*, are homologous to the *E. coli hlyB* and *hlyD* genes which are responsible for secreting Hly in *E. coli* (4, 20, 25). Previous data suggest that *cyaB* and *cyaD* may be functioning similarly in *B. pertussis* in transporting the AC toxin (9, 15). We made mutations in these downstream sequences to evaluate their role in secretion.

BPK355 was constructed by deleting a 2.1-kilobase *Pst*I fragment and inserting a kanamycin resistance gene. Returning this construct to the chromosome deleted segments of *cyaB* and *cyaD;* it may also block expression of the downstream gene *cyaE* (Fig. 1) if, as is suggested by sequence and transcriptional analysis, an operon structure exists (9, 13). BPK355 was nonhemolytic on blood agar plates and was deficient in AC enzyme and toxin activities (Table 2). A whole-cell preparation of BPK355 had no enzyme activity; the sonicated preparation had 4% wild-type activity. This result indicates a compartmentalization defect in this mutant. The mutant toxin does not appear to be located in the normal extracytoplasmic, cell-associated location. This finding supports a role for the downstream genes in transport.

Western blot analysis demonstrated that less AC toxin protein was being made in this mutant. The presence of only 4% of wild-type activity would indicate that downstream genes may be necessary for stabilizing the structural gene transcript or product.

The most distal open reading frame, *cyaE,* has no *E. coli* homolog (9). A mutation was created in *cyaE* by integrating the plasmid pEB317 at the *Sau*3A site by homologous recombination as above (Fig. 1). This mutant, BPR327, is the result of a single recombination event. BPR327 was nonhemolytic on blood agar plates and deficient in AC toxin and enzymatic activities. Whole-cell preparations of BPR327 had 3% wild-type enzymatic activity; a sonicated preparation had 10% wild-type activity (Table 3). Although whole-cell activity is much reduced from that of the wild type, enzyme activity is present, indicating that *cyaE* may not be playing a role in secretion. The reduced activities may again reflect a stabilization function.

Animal studies. The infant mouse model of pertussis infection has been used to evaluate the contribution of individual virulence factors in disease (22). In the present study, the researchers evaluated the ability of the AC toxin mutant to cause lethal infections upon intranasal infection in the 6-day-old mouse (Fig. 2). The parental strain SM001 caused a lethal infection at a dose of 5.4 x 10^4 CFU. In contrast, the mutants in *cyaA* required a higher dose to cause a lethal infection. Strain BP358 was lethal at a dose of 4.5 x 10^7 CFU. At this dose strain, BP377 was still not lethal. These results demonstrate that the mutant proteins which retain enzymatic activity but lack toxin and hemolytic activities were not effective in contributing to the virulence of *B. pertussis* in mice.

TABLE 3. AC and hemolytic activity in downstream mutants

Strains	Hemolytic activity[a]	AC toxin activity[b]	AC[c]	
			Whole cells	Lysed cells
SM001	+	+	100	100
BPK355	–	–	0	4
BPR327	–	–	3	10
BP348	–	–	0	0

[a] Hemolytic activity was assayed on blood agar plates.
[b] Toxin activity was measured in urea extract preparations.
[c] Enzyme activities are expressed as a percentage of wild-type activity in strain SM001.

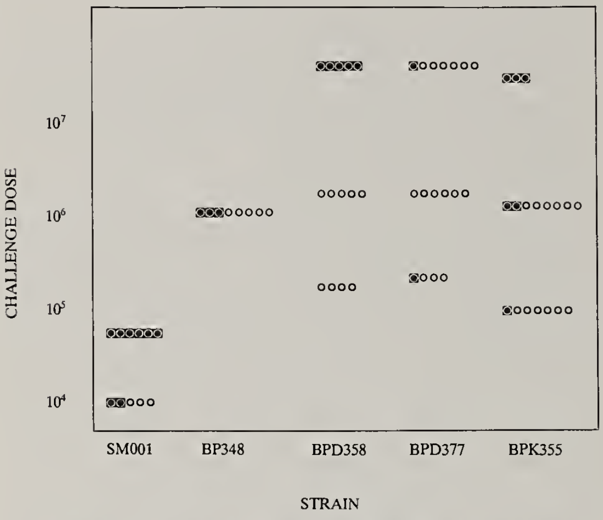

FIG. 2. Challenge of infant mice with mutant *Bordetella* strains. The number of symbols indicates the number of mice challenged at that dose. Closed symbols indicate that the mice died; open symbols indicate that the mice survived the challenge.

The invasive nature of the toxin appears to be necessary for disease; enzyme activity alone was not sufficient.

BPK355 was avirulent at the wild-type lethal dose and was not fully virulent until a dose of 3.4 x 10^7 CFU was used. This result indicates that if the toxin is not stabilized in its normal location, it may not be available as a virulence factor. Alternatively, not enough toxin may be made in the mutants to be virulent.

DISCUSSION

Full AC toxin activity includes several actions of a single molecule plus the action of other gene products. The single protein must possess a catalytic domain as well as domains which will bind targets and allow delivery of the catalytic site to the target cell cytoplasm. The catalytic domain has previously been defined (9), and the researchers have shown that mutations in the structural gene downstream from this domain do not affect enzyme activity. These structural gene mutations do define regions that are necessary for toxin and hemolytic activities. The lack of activity in the deletion mutants may be explained by the loss or disruption of a domain responsible for target binding or internalization. The interaction of the AC toxin and Hly with target cells has not been defined. A first step towards understanding this association will be to test these mutants for target-binding ability.

Toxin and hemolytic activities require the structural gene product to be activated by the *cyaC* gene product. The *cyaC* mutant has full enzyme activity but no toxin or hemolytic activity; this is the same phenotype as the structural gene mutants described here. An alternative explanation for the lack of activity in the *cyaA* mutants is that the domain within the structural protein on which the *cyaC* gene product acts was deleted or disrupted.

Interestingly, toxin and hemolytic activities were not separable in these mutants. The relationship between these two sets of activities is not fully understood, nor is it known which is responsible for the virulence capacity of AC toxin –Hly. Further, it is not known if hemolytic activity requires enzymatic activity or is the result of membrane perturbation.

Sequences downstream from *cyaA* have been implicated in the transport of the AC toxin both by sequence analysis and homology to *E. coli* secretion genes and by complementation studies (8, 15). The downstream mutants described here support a transport role for these genes. The study mutants also introduce the idea that the molecule may not be stable if it is not transported to its correct location. A more site-specific mutagenesis strategy for each of the downstream genes will be required to resolve the role played by each.

Although several factors play a role in whooping cough, clearly the AC toxin is an essential element. Animal studies with the *cyaA* mutants demonstrate that enzyme activity alone is not sufficient for virulence in the mouse model. The mutants lacking toxin and hemolytic activities were avirulent. Such avirulent strains may be considered for vaccine applications since it has been shown that they produce the AC protein which reacted with monoclonal antibody in a Western blot. The strains are therefore immunogenic and nontoxic.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-23695 from the National Institute of Allergy and Infectious Diseases, and by a PEW Foundation Fellowship to A.A.W. and National Institutes of Health grant AI-18000 to E.L.H.

The authors gratefully acknowledge the contributions of Donna Taylor, Martha McLeod, and George Vandenhoff (University of Virginia Diabetes Center RIA Core Laboratory) for cyclic nucleotide assays.

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DISCUSSION

DR. ULLMANN: This is fantastic work, and it is really a beautiful to have the C-gene also in pertussis. I am very glad for that. Is it *bvg* regulated, the C-gene?

MS. BARRY: That has not been determined yet. We are working on that right now.

Three Sites Upstream of the *cya* Operon Are Required for Phenotypic Modulation and Hemolytic Activity of the Adenylate Cyclase from *Bordetella pertussis*

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Many pathogenic bacteria have developed a survival strategy that relies on a signal response mechanism in which the bacteria monitor and respond to the environment with the expression of different phenotypes depending on environmental conditions. This process, known as phenotypic modulation, is controlled by a signal transduction mechanism which regulates gene expression at the level of transcription (for a review, see references 6 and 20). *Bordetella pertussis* responds to environmental stimuli by global regulation of a number of virulence factors including pertussis toxin, filamentous hemagglutinin, and the extracellular calmodulin-sensitive adenylate cyclase. These virulence genes are coordinately regulated by the *bvg* locus originally called *vir* (21, 24, 32, 33). This locus contains two contiguous genes, *bvgA* and *bvgS* (2; S. Stibitz, personal communication), with predicted protein sequences homologous to the superfamily of signal transducing pairs of proteins (sensor/regulator) also known as the two-component system for signal transduction (for a review, see references 1 and 31). The protein products of the *bvg* locus may regulate gene transcription via the putative DNA binding domains identified on both *Bvg* A or S as described for other homologs of the two component system (1, 30). In culture, phenotypic modulation of virulence factors in *B. pertussis* can be induced by an increase in $MgSO_4$, nicotinic acid, or a decrease in temperature (12, 14, 23). This is caused by a bvg-mediated decrease in gene transcription of these virulence factors (15, 19).

The *bvg*-regulated *cya* operon is the genetic locus that encodes the adenylate cyclase hemolytic phenotype (Cya/Hly). It is composed of four contiguous genes: *cyaA*, *B*, *D*, and *E*. The *cyaA* encodes the structural gene for which protein contains both adenylate cyclase and hemolytic activities, while *cyaB*, *D*, and *E* encode proteins required for secretion (9, 10; for a review, see reference 11). The predicted protein sequences of *cyaA*, *B*, and *D* display a large degree of similarity to the predicted protein sequences of *hlyA*, *B*, and *D* genes of the *hly* operon which is responsible for the hemolytic phenotype in *Escherichia coli* (10). The *hly* operon contains an additional *hlyC*, upstream from *hlyA*, that is required for the hemolytic phenotype and presumably covalently modifies *HlyA*, activating its hemolytic activity (22).

The promoter for the *cya* operon has been identified by primer extension analysis, and several studies have shown that the Hly/Cya phenotype is regulated by the *bvg* locus and that regulation is at the level of gene transcription (15, 34). The possible regulatory sites in the upstream region of the *cya* operon have not been identified, however. In this study, a new technique was developed to deliver transposon insertions to a defined chromosomal region upstream of the *cya* operon. Two genetic elements were identified upstream from the promoter of the *cya* operon that are responsible for phenotypic modulation of the *cya* operon presumably by the *bvg* locus. A third region was also identified by transposon mutagenesis, complementation analysis, and inspection of the DNA sequence; this region suggests that an additional gene, presumably *cyaC*, is responsible for conferring the hemolytic activity to the adenylate cyclase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains of bacteria, plasmids, and lambda phage used in this study are summarized in Table I. *B. pertussis* was grown on Bordet-Gengou (Difco Laboratories, Detroit, Mich.) agar plates containing 10% sheep blood or in

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Presented by Elaine Tuomanen.

TABLE 1. Bacterial strains and plasmids

Strains, plasmids, and phage	Description	Source or reference
E. coli		
DH5α	F^-φ80d*lac*ZΔ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (r_K–m_K+)*sup*E44λ– *thy*-1 *gyr*A *rel*A1	Bethesda Research Laboratories
CC118	*ara*D139 Δ(*ara leu*)7697 Δ*lac* X74 *pho*A20 g*al*E *gal*K *thi* *rps*E*rpo*B *arg*I(Am) *rec*A1	17
ECRU 21	DH5α containing pUC with 2.3-kb *Bam*HI *B. pertussis* fragment	This study
ECRU 22	DH5α containing pKMS 1; pSORTP1 with *Bam*HI *B. pertussis* fragment	This study
ECRU 23	CC118 containing pKMS 1; pSORTP1 with 2.3-kb *Bam*HI *B. pertussis* fragment	This study
ECRU 25	CC118 containing pKMS 2; pLAFRx with 2.3-kb *Bam*HI *B. pertussis*	This study
MM294 (pRK 2013::Tn9)	*end* A, *hsd* R, *pro* *sup* F with pRK 2013::Tn*9*	8
B. pertussis		
BPRU 00	Tohoma phase I spontaneous mutant Sm^r *bvg*$^+$	This study
BPRU 20	BPRU 00, *cya*::Tn*5tac1*	This study
BPRU 44	BPRU 00, *cya*::Tn*5tac1*	This study
BPRU 46	BPRU 00, *cya* C::Tn*5tac1*	This study
BPRU 65	BPRU 20 with pKMS 2	This study
BPRU 66	BPRU44 with pKMS 2	This study
BPRU 67	BPRU46 with pKMS 2	This study
Plasmids		
pLAFRx	Tc^r, pRK290 derivative with a multiple cloning site	8, G. Weinstock
pSORTP1	Ap^r, Gt^r, Sm^s; gene replacement vector	29
pRK 2013::Tn9	IncP1 *tra ori*E1 Tn9 Cm^r	
pUC18Cm	Cm^r; multiple cloning vector	T. Davis
pHRM 23	pUC18Cm with 2.3-kb *Bam*HI fragment from *B. pertussis*	This study
pKMS 1	pSORTP1 with 2.3-kb *Bam*HI fragment from *B. pertussis*	This study
pKMS 2	pLAFRx with 2.3-kb *Bam*HI fragment from *B. pertussis*	This study
Phage		
λ::Tn*5tac1*	*b*221 *c*I857 *O*am29 *P*am80::Tn*5tac1*	Chow and Berg

liquid medium described by Stainer and Scholte (28). Antibiotics, when needed, were added at the following concentrations: kanamycin, 40 µg/ml; streptomycin, 300 µg/ml; and tetracycline, 12.5 µg/ml. *E. coli* was grown in L broth or L-agar plates (27). Antibiotics for this bacterium were added at these final concentrations: ampicillin, 100 µg/ml; kanamycin, 40 µg/ml; and tetracycline, 12.5 µg/ml. When indicated, isopropyl-ß-D-thiogalactoside (IPTG) was added to liquid cultures of *B. pertussis* (OD_{620}; 0.3) at 150 µg/ml and harvested 3 h later.

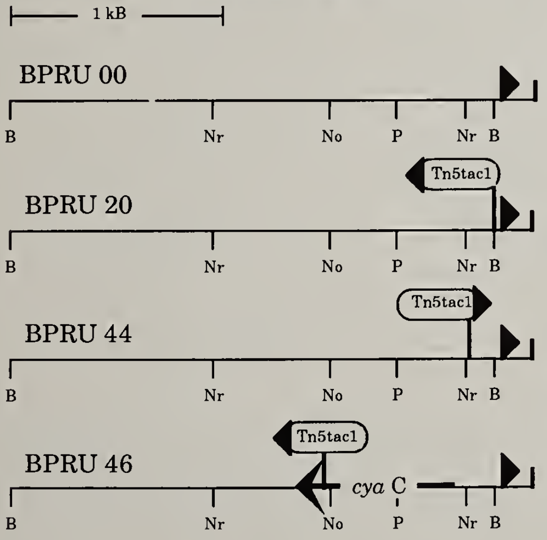
Recombinant DNA methods. Standard methods were used for the isolation of plasmid and chromosomal DNA, restriction enzyme analysis of plasmids, hybridization of specific radioactive DNA probes with chromosomal DNA, agarose gel electrophoresis, and DNA ligations (26). Plasmid DNA probes were labeled with ^{32}P using a nick-translation kit (New England Nuclear Corp., Boston, Mass.).

Transposon mutagenesis and conjugative transfer of plasmid DNA from *E. coli* to *B. pertussis*. Transposon mutagenesis of plasmids containing chromosomal DNA from *B. pertussis* was performed in *E. coli* as described elsewhere (5). These mutagenized plasmids were mated into *B. pertussis* with *E. coli* strain CC118 as the donor strain and MM294 (pRK 2013) containing the transfer functions as the helper strain. Matings were performed as described elsewhere (29).

Adenylate cyclase assay and detection of hemolysis. The production of cAMP was measured by the method described by Salomon et al. (25). Samples were assayed in the presence of 2.5 µM of calmodulin. Bacterial-induced hemolysis was detected as a clear zone surrounding bacterial colonies grown on Bordet-Gengou plates containing 10% sheep blood.

Cell fractionation and proteolysis. Extracellular adenylate cyclase activity was detected in bacterial-cell-free culture supernatants. Intracellular adenylate cyclase was detected in trypsin-treated cells that had been lysed,

Restriction map and positions of transposon insertions in the 2.3 kb region upstream of the cya operon [a]



Adenylate cyclase activity [b]				***Hemolytic colonies*** [c]	
w/o $MgSO_4$		w/ $MgSO_4$		$MgSO_4$	
Culture Supernatant	Intracellular	Culture Supernatant	Intracellular	w/o	w/
1.39 ±.09	0.101 ±0.01	0.00	0.00	+	○
2.22 ±.13	0.146 ±0.01	2.78 ±.02	0.126 ±0.01	+	+
0.00	.011 ±0.01	0.00	0.102 ±0.01	○	○
0.58 ± 0.3	N.D.	0.00	N.D.	○	○

FIG. 1. Characterization of the transposon mutations upstream from the *cya* operon. Southern analysis was used to characterize the Tn*5tac1* transposon insertions within the 2.3-kb fragment upstream from the *cya* operon in three mutant strains of *B. pertussis*. BBRU 00 is a spontaneous Str^r mutant of Tohama phase I. BPRU 20, 44, and 46 containing Tn*5tac1* insertions in the upstream region of the *cya* operon were created as described in text. Symbols for restriction enzyme sites are: B, *Bam*HI; Nr, *Nru*I; No, *Not*I; and P, *Pst*I. (▲) indicates the presence and direction of promoters. Bacterial cultures were grown in Stainer-Scholte medium in the presence (w/) or absence (w/o) of 30 mM of $MgSO_4$ to an OD_{620} of 0.5 and the bacterial-cell-free culture supernatants and intracellular fractions collected as described in text. These samples were assayed for the ability to produce cAMP and the concentration of protein. Values are presented as µmol of cAMP/min per mg of protein. Hemolysis for the different strains was scored as the ability of individual colonies to form a clear concentric zones on Bordet-Gengou 10% blood agar plates in the presence or absence of 40 mM $MgSO_4$.

sonicated, and fractionated by centrifugation to isolate the soluble intracellular proteins. This procedure has been described elsewhere (18).

RESULTS

Cloning and shuttle transposon mutagenesis of the upstream region of the *cya* operon. The *cya* operon of *B. pertussis* is composed of an 11-kilobase (kb) fragment of DNA that contains four contiguous genes, *cyaA*, *B*, *D*, and *E*. A 2.3-kb *Bam*HI-*Bam*HI fragment 39 base pairs (bp) upstream from the promoter of the *cya* operon was isolated from clone 1 of a λgt 11 library from *B. pertussis* described previously (Au et al., 1989). This fragment was cloned into pUC18Cm (pHRM23); a restriction map of this fragment is presented in Fig. 1.

In an effort to identify regions upstream from the *cya* operon required for gene expression, a strategy was developed for delivering transposon mutations to this defined region and returning these mutations to the *B. pertussis* chromosome via a gene replacement vector pSORTP1 (Stibitz et al., 1986). This fragment was subcloned into the *Bam*HI site of pSORTP1. An *E. coli* strain, ECRU 23, bearing the resulting plasmid pKMS 1 was subjected to transposon mutagenesis with Tn*5tac1*.

A pool of these insertion mutants was mated back into a Str^r strain of *B. pertussis* and Kan^r, Str^r exconjugants were selected on Bordet-Gengou blood agar plates. The surviving exconjugants are those that have undergone a double crossover event incorporating the Kan^r transposon into the chromosome within the 2.3-kb region. Two hundred exconjugants were scored for the hemolytic phenotype. Six mutants were Hly$^-$ on blood agar plates; two of these were further characterized (BPRU 44 and 46). The Hly$^+$ mutants were streaked on Bordet-Gengou blood agar plates supplemented with 30 mM of $MgSO_4$ to encourage phenotypic modulation of the virulence factors. Under these conditions, four of these mutants expressed the Hly$^+$ phenotype while the remaining mutants underwent phase modulation and were Hly$^-$. One of these Hly$^+$ mutants was further characterized (BPRU 20).

Chromosomal mapping of the transposon insertions. Tn*5tac1* contains an inducible outward-facing promoter, P_{tac}, near one end of the transposon (5). Therefore, it was important to map the orientation as well as the position of the transposon insertion in each of the mutants. Figure 1 summarizes the results of Southern analysis of the transposon insertions. The transposon in BPRU 20 inserted near the *Bam*HI site 39 bp upstream of the *cyaA* promoter with P_{tac} facing away from the *cyaA* promoter. The transposon in BPRU 44 mapped between the *Nru*I and *Bam*HI sites with P_{tac} facing the

TABLE 2. Complementation of the transposon mutations in the upstream region of the *cya* operon

Strain[a]	Description	Adenylate cyclase activity[b]		Hemolytic colonies[c]	
		40 mM $MgSO_4$		40 mM $MgSO_4$	
		w/o	w/	w/o	w/
BPRU 00	Isogenic parent	1.50 ± 0.10	0	+	0
BPRU 65	BPRU 20 with pKMS 2	2.30 ± 0.09	2.6 ± 0.20	+	+
BPRU 66	BPRU 44 with pKMS 2	0	0	0	0
BPRU 67	BPRU 46 with pKMS 2	1.60 ± 0.11	0	+	0

[a] pKMS 2, which contains the 2.3-kb fragment upstream from the *cya* operon, was conjugated into the various transposon insertion mutants of *B. pertussis*.

[b] Mutant strains of *B. pertussis* were grown to an OD_{620} of 0.5 either with (w/) or without (w/o) 40 mM of $MgSO_4$ added to the growth media as described in the legend for Fig. 1. The bacteria-free culture supernatant was isolated and assayed for the ability to produce cAMP. Values are presented as µmol cAMP/ min per mg of protein.

[c] Hemolytic colonies were determined for each strain as described in the legend for Fig. 1.

```
Cya C GQYILLRCNDVPVAYCSWALMNDADTELSYVMAPSSLGGNAWNCGDRLWLIDWIAP
      .::.::  .. :::::::: ..  ..:..:. . .::  ..:..::: :.::::::
Hly C NQYVLLTRDNYPVAYCSWANLS-LENEIKYLNDVTSLVAEDWTSGDRKWFIDWIAP


Cya C  FSRDDNRALRRALAERAPDSVRGRSLRVRRGGDTARVKEYRGRALD
       :  .:: :: . . .. ::..   ..::  . ....:.:..:  .:
Hly C  F--GDNGALYKYMRKKFPDELF-LAIRVDPKTHVGKVSEFHGGKID
```

FIG. 2. Comparison of the predicted protein sequences of Cya C and Hly C. The predicted amino acid sequence of Cya C (amino acid 52-153) was aligned with the predicted amino acid sequence of Hly C (amino acid 42-139) (7) using the Fastp align program developed for an Apple Macintosh (16). There is a 40% identical sequence identity in this region. It has been proposed that *cya* C is in the opposite orientation to the *cya* operon (Barry et al., submitted) (Fig. 1).

*cya*A promoter. The transposon in BPRU 46 mapped between a second *Nru*I site, close to the *Not*I site with P_{tac} facing away from the *cya* promoter.

Characterization and localization of the adenylate cyclase activity. Phase modulation of the *cya* operon is under control of the *bvg* locus. Addition of 40 mM of $MgSO_4$ to the growth medium of wild-type bacteria inhibits adenylate cyclase production at the level of gene transcription (15, 34). The amounts of extra- and intracellular adenylate cyclase activity for the mutants 20, 44, and 46 were compared with that of the isogenic parent in the absence and presence of $MgSO_4$ (Fig. 1). BPRU 20 constitutively produced extra- and intracellular adenylate cyclase activity regardless of the presence of $MgSO_4$. The amount of adenylate cyclase activity produced by cultures of BPRU 20 was 60% higher than the isogenic parent when grown to an identical OD_{620} of 0.5 in Stainer-Scholte medium. The insertion into this region 39 bp upstream from the *cya* promoter suggests that the disrupted region represses transcription of the *cya* operon and acts as either a *cis*-acting repressor or as an element that binds a *trans*-acting repressor.

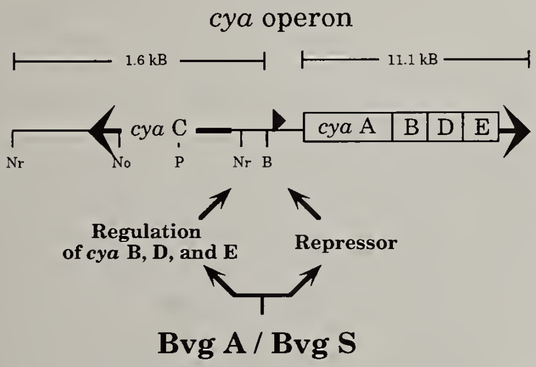


FIG. 3. Genetic organization of the *cya* operon and proposed model for phenotypic modulation. Based on characterization of transposon insertion mutations in the upstream region of the *cya* operon, there are two sites for phenotypic modulation by the *bvg* locus that lie in the 116 bp flanked by the *Nru*I and *Bam*HI restriction sites. The region at the *Bam*HI site is either a *cis*-acting repressor or binds a *trans*-acting repressor. The *bvg* locus activates the operon by counteracting this repressor. A second site 150 bp upstream from the *cyaA* promoter may be necessary for *bvg* regulation of the expression of the *cyaB*, *D*, and E genes required for secretion. There is an additional region that contains the gene *cyaC* that is responsible for the activation of the hemolytic phenotype associated with CyaA, the adenylate cyclase toxin. The restriction enzyme site designations are the same as in Fig. 1. (▸) indicates the position of the *cyaA* promoter.

BPRU 44 produced only an intracellular enzyme activity that was not regulated by $MgSO_4$. This mutant produced 35% more enzyme activity when grown in the presence of 150 μg/ml of IPTG; in contrast, the parent strain and the other mutants did not exhibit a similar increase (data not shown). This finding suggests that an insertion approximately 150 bp upstream from the *cya* promoter disrupted secretion but not production of the adenylate cyclase. It is possible that the *cya* promoter is no longer functional and that transcription is originating from P_{tac} even in the absence of IPTG.

Even though colonies of BPRU 46 were not hemolytic, they did produce an extracellular adenylate cyclase activity that was regulated by the addition of $MgSO_4$ to the growth medium. This suggests that an insertion 1.1 kb upstream from the promoter of *cyaA* gene allowed expression and secretion of CyaA in a *bvg*- regulated manner, but the hemolytic activity associated with the adenylate cyclase was lost.

Complementation of the mutant phenotypes. A vector was constructed containing the 2.3-kb sequence cloned into the *Bam*HI site of the shuttle vector pLAFRx.

A strain of *E. coli* (ECRU 25) harboring the resulting plasmid, pKMS 2, was mated with BPRU 20, 44, and 46. Tet^r, Kam^r exconjugants were selected, and a single colony from each mating was purified and characterized.

The production of adenylate cyclase activity and the presence of hemolytic colonies in these mutants are summarized in Table 2. Complementation in *trans* of the insertion in mutant BPRU 20 did not restore the *bvg*-mediated response to $MgSO_4$ (BPRU 65). The parental phenotype was also not restored in BPRU 44 when complemented by the plasmid-borne sequence supplied in *trans* (BPRU 66). This result contrasted with the insertion mutation present in BPRU 46: the parental Cya/Hly phenotype was restored when the 2.3-kb plasmid-borne sequence was supplied in *trans*, suggesting that this sequence encodes a *trans*-acting factor that restores hemolytic activity associated with the adenylate cyclase toxin. Inspection of the published nucleotide sequence (9) of part of the 2.3-kb fragment (*Not*I to the *Bam*HI site) reveals a reading frame (*cyaC*) in the opposite orientation to the *cya* operon that has a large degree of predicted protein sequence similarity to HlyC (Fig. 2). The gene product of the *hlyC* is responsible for the covalent modification of hemolysin in *E. coli* and thus the hemolytic phenotype (22). This work is in agreement with that of others (E. M. Barry, A. A. Weiss, I. E. Ehrmann, M. Grey, E. L. Hewlett, and M. Goodwin, submitted for publication) who also identified an open reading frame (*cyaC*) in the opposite orientation to the *cya* operon.

DISCUSSION

In this study a new technique was developed to direct transposon insertions to a defined area of the *B. pertussis* chromosome. The upstream region of the *cya* operon from *B. pertussis* was studied to define specific sites required for gene expression. Transposon mutagenesis was first directed to an upstream sequence in *E. coli*. Transposon insertion mutations were then delivered from *E. coli* back to the *B. pertussis* genome via conjugation with a nonreplicating suicide vector. This technique can be termed shuttle transposon mutagenesis. A similar strategy has been employed for isolating the transformation genes in *Haemophilus influenzae* (31).

With this technique, two regions upstream of the *cya* operon were identified that alter phenotypic modulation of the expression of this operon by the *bvg* locus. A third region was identified that contains a new gene, *cyaC*. Disruption of this gene dissociates the hemolytic from the adenylate cyclase activities of the toxin molecule. The genetic organization of the *cya* operon and a proposed model for phenotypic modulation are presented (Fig. 3).

Analysis of mutants in the intergenic region between *cyaC* and *cyaA* suggests that phenotypic modulation by the *bvg* locus is not a simple, direct activation. Two genetic elements influencing adenylate cyclase production and secretion lie within the 116-bp sequence between the *Nru*I and *Bam*HI restriction sites 39 bp upstream from the *cyaA* promoter. These elements are a repressor element and a regulatory site for the expression of *cyaB*, *D*, and *E*. Both regions are linked to *bvg* regulation of the operon. The repressor region was defined by insertion of a transposon 39 bp upstream from the *cyaA* promoter, producing a constitutive mutant under modulating conditions (high $MgSO_4$), i.e., conditions that were no longer *bvg* regulated and not complemented by the sequence in *trans*. It is proposed that the region 39 bp upstream from the *cya* operon is required for *bvg* regulation of the *cya* operon, and that this element acts as either a *cis*-acting repressor or as an element that binds a *trans*-acting repressor. Furthermore, *bvg* regulation appears to involve inactivation of the repressor activity and not activation of transcription of the *cya* operon. This activity could occur either directly via the DNA-binding domains of *bvgA* and *bvgS* or through some as yet unknown intermediate. This mutation is to be distinguished from a similar mutation to the *mod* locus (13) which presumably maps to the *bvg* operon (6) and constitutively expresses all of the virulence genes under modulating conditions.

A regulatory site for expression of *cyaB*, *D*, and *E* was defined by a transposon insertion in the region 150 bp upstream from the *cyaA* promoter. This mutation permitted *cyaA* expression, but disrupted secretion of CyaA as well as *bvg* regulation of the *cya* operon. The mutation was not complemented with the sequence provided in *trans*. The transcription of *cyaA* in this mutation may be due to the presence of P_{tac} from the transposon. It is proposed that this region is required for *bvg*-mediated phenotypic regulation and for the transcription of the genes required for secretion, presumably, *cyaB*, *D*, and *E*. This hypthesis contrasts with that of another report that suggests that there is constitutive expression of *cyaB*, *D*, and *E* under modulating conditions (15).

Finally, a transposon insertion 1.1 kb upstream from the promoter of the *cyaA* gene (BPRU 46) allowed expression and secretion of CyaA in a *bvg*-regulated manner; however, the hemolytic activity associated with CyaA was lost. It is proposed that this region contains an additional *cyaC* whose gene product presumably covalently modifies the adenylate cyclase conferring hemolytic activity. This hypothesis is supported by two findings. First, the insertion mutation was complemented in *trans* with a plasmid bearing this sequence. Second, a reading frame in the opposite orientation to the *cya* operon presumably encodes a putative protein that shows extensive sequence similarity to HlyC, a protein required to confer hemolytic activity to the hemolysin from *E. coli* (22).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants RO1 AI23459 and RO1 AI27913 from the National Institutes of Health and a Career Scientist award from the I. T. Hirshl Trust.

The authors thank Dan Storm (University of Washington, Seattle, Wash.) for providing clone 1 from the *B. pertussis* λ GT11 library, Terry Davis (University of Washington) for pUC18Cm, Colin Manoil (University of Washington) for *E. coli* strain CC118, George Weinstock (University of Texas, Houston, Tex.) for pLAFRx, David Relman (Stanford University, Stanford, Calif.) for pSORTP1, and Doug Berg (Washington University, St. Louis, Mo.) for λ::Tn*5tac1*. The authors also thank Elaine Tuomanen (Rockefeller University, New York, N.Y.) for many helpful discussions and a careful reading of this manuscript.

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DISCUSSION

DR. ROY: I would like to say that the field of regulation is pretty confusing right now, and there is a lot of alternative explanations which can explain these data, and first of all there is the repressor. Unless the transcriptional start site has been mapped in that mutant, I would say it is more likely that that mutation is due to a Tn*5* promoter or formation of a cryptic promoter through the insertion of Tn*5* because insertion of Tn*5* in a repressor binding site would be more likely to destroy the promoter since repressor binding sites often overlap essential elements for that promoter.

DR. TUOMANEN: I think that Rob has appreciated some of your comments and is in the process of making fusions of these upstream regions with other gene products so he can assess how they in fact will determine downstream gene production and that would address precisely your types of questions. But there is a significant distance between this mutation and the promoter so we are talking about something like 100 base pairs up.

DR. ROY: That would even be more unusual for repressor base test of 100 base pairs upstream of the promoter which would be very interesting.

DR. STIBITZ: As a point of clarification, do I understand you to say that that mutation interrupts the structural gene for repressor or is an operator?

DR. TUOMANEN: I do not think we know anything about exactly how it works at all. Right now it is a transposon phenotype.

DR. ROY: And I guess this would go to Brad as how leaky is Tn*5tac* because it seems likely that that other mutation would be due to leaky read-through of Tn*5tac* and reading into the cyclase gene which you probably would not need a lot of to get activity.

DR. TUOMANEN: The second mutation, it is possible to interpret that way, and, in fact, if you put in IPTG, you get about a 30 percent increase in the production of the *cya* gene product. The interesting part is that you do not go beyond that into the BDE region and so that it does remain possible that because the Tn*5tac* is oriented towards the *cyaA* gene that it is the promoter that is driving that production.

DR. STIBITZ: A possibly related question. Can you comment on the constitutive level of activity? Is it the same as wild-type induced levels?

DR. TUOMANEN: As I understand it, the constituative level is approximately 30 percent higher than wild type levels.

P.69 Pertactin: High-Level Expression and Purification from *Escherichia coli* and Protective Properties

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The vir-regulated protein P.69 pertactin from *Bordetella pertussis* is a member of an immunologically cross-reactive family of proteins represented in *B. parapertussis* by a 70-kDa antigen (P.70 pertactin) and in *B. bronchiseptica* by a 68-kDa antigen (P.68 pertactin) (11, 12). The *B. pertussis* P.69 protein is located on the bacteria's outer membrane (12); it has also been identified as an agglutinogen (2). Recent studies showed that this family of antigens exhibits eukaryotic cell-binding activity which is mediated, at least in part, by the Arg-Asp-Gly sequence present within the mature (i.e., fully processed) amino acid sequence (3, 6). Consequently, the name "pertactin" has been proposed for this family of proteins (6).

Immunizing mice with purified P.69 from *B. pertussis* results in their immunity to aerosol challenge with virulent *B. pertussis* (15; M. Roberts, N. Fairweather, and I. Charles, submitted for publication). Passively administered monoclonal antibodies reactive against P.69 or P.68, respectively, have protected against *B. pertussis*- and *B. bronchiseptica*-mediated disease. (12, 15). In addition, Kobish and Novotny (5) used purified P.68 to immunize pregnant sows and found that the offspring had high anti-P.68 antibody titers and were protected against aerosol challenge of *B. bronchiseptica*. These data demonstrate that the pertactin family of proteins is important in the development of immunity to diseases caused by the *Bordetellae*; they further suggest that P.69 pertactin may be an important component of acellular vaccines against whooping cough in children.

Charles et al. previously reported on the cloning and characterization of the gene for P.69 (3) and demonstrated that it encodes a protein with a calculated molecular mass of 93 kDa. This protein is processed in vivo by removing a signal peptide and a C-terminal fragment to yield a molecule with an apparent mass of 69,000. *B. pertussis* produces P.69 at low levels, and many liters of culture must be grown to purify large amounts of antigen. Also *B. pertussis* produces many virulence factors, raising the possibility of P.69 contamination with e.g., pertussis toxin. This study therefore set out to express high levels of P.69 in *Escherichia coli* and to characterize the properties of the recombinant protein.

MATERIALS AND METHODS

Construction of recombinant plasmids. Plasmid pPERtacl was constructed by cloning synthetic oligonucleotides encoding the amino-terminal region of P.69 and the carboxy-terminal region around amino acid residues 544, 619, and 649 into the expression plasmid pTETtac1 (7). Plasmids pPERtac2, 3, 4, 7, and 8 were all constructed by cloning various *B. pertussis* P.93 coding sequences obtained from pMLU1, a subclone of pBPI69 (3) into pPERtac1. All DNA manipulations were as described by Maniatis et al. (10). Further construction details are provided elsewhere (A. J. Makoff, M. D. Oxer, S. Ballantine, N. F. Fairweather, and I. G. Charles, Biotechnology, in press).

Induction and analysis of expressed proteins. All expression plasmids contain a functional copy of the *lacI* gene and can therefore be induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures of *E. coli* MM294 containing the plasmids were induced as described previously (7,8); P.69-specific proteins were detected by Western blotting using anti-P.69 antibody (11). P.69 from *B. pertussis was purified* as described previously (12).

Purification of P.69 from *E. coli*. *E. coli* MM294 (pPERtac36) was grown overnight in L-broth (10) and diluted 1 in 5 in a fresh medium containing 60 mM IPTG.

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* Presenting author.

Cells were grown for 4 h and harvested by centrifugation (10,000 x g, 10 min). The cells were resuspended in 50 mM Tris-HCl pH 7.5, 50 mM NaCl, and lysed by two passages through a French pressure cell (15,000 lb/in^2). The pellet fraction containing insoluble P.69 was washed in buffer A (50 mM Tris-Hcl pH8.0, 100 mM NaCl, 1 mMEDTA) containing 1% Triton X-100 and centrifuged as before. To solubilize the pellet, it was resuspended in buffer A containing 6 M GuHCl. The protein concentration was adjusted to 6 mg/ml and the GuHCl molarity reduced to 1 M by dilution. The solution was dialysed at 4°C for 24 h with three changes against buffer A and the insoluble precipitate which appeared on dialysis was removed by centrifugation at 10,000 g for 10 min.

Intercerebral challenge test. Vaccines were made up in 10% alhydrogel in PBS. Fourfold dilutions of 0.5 ml were injected subcutaneously into male and female NIH(S) mice. After 14 days, the mice were challenged intercerebrally with 20μl of *B. pertussis* 18323 (approximately 50 LD_{50}s); the survivors were counted 14 days later.

RESULTS

Identification of the C-terminus of P.69. Charles et al. described the expression of fragments of P.69 in *E. coli*, including one plasmid which expressed the entire structural gene, excluding the first 24 amino acids, for P.93 (3). That study estimated the C terminus of P.69 to be around amino acid residue 690 based on calculating the molecular mass of proteins translated from the DNA sequence and the mobility of P.69 on SDS-PAGE gels. To locate the C terminus of P.69 more accurately, we constructed a family of plasmids in *E. coli* which expressed a nested set of proteins with different C termini (Fig. 1). The signal sequence was omitted from these recombinant proteins, and so the first amino acid after the methionine initiation codon is Asp-1, which is the first residue of the mature P.69 (3). The proteins expressed were identified by Western blotting of *E. coli* whole-cell lysates using the anti-P.69 antibody (Fig. 2). Plasmids pPERtac3 and 4 were constructed to produce proteins of 62.5 and 66 kDa which were expected to migrate faster on SDS-PAGE than P.69. However, these plasmids actually expressed proteins which migrated

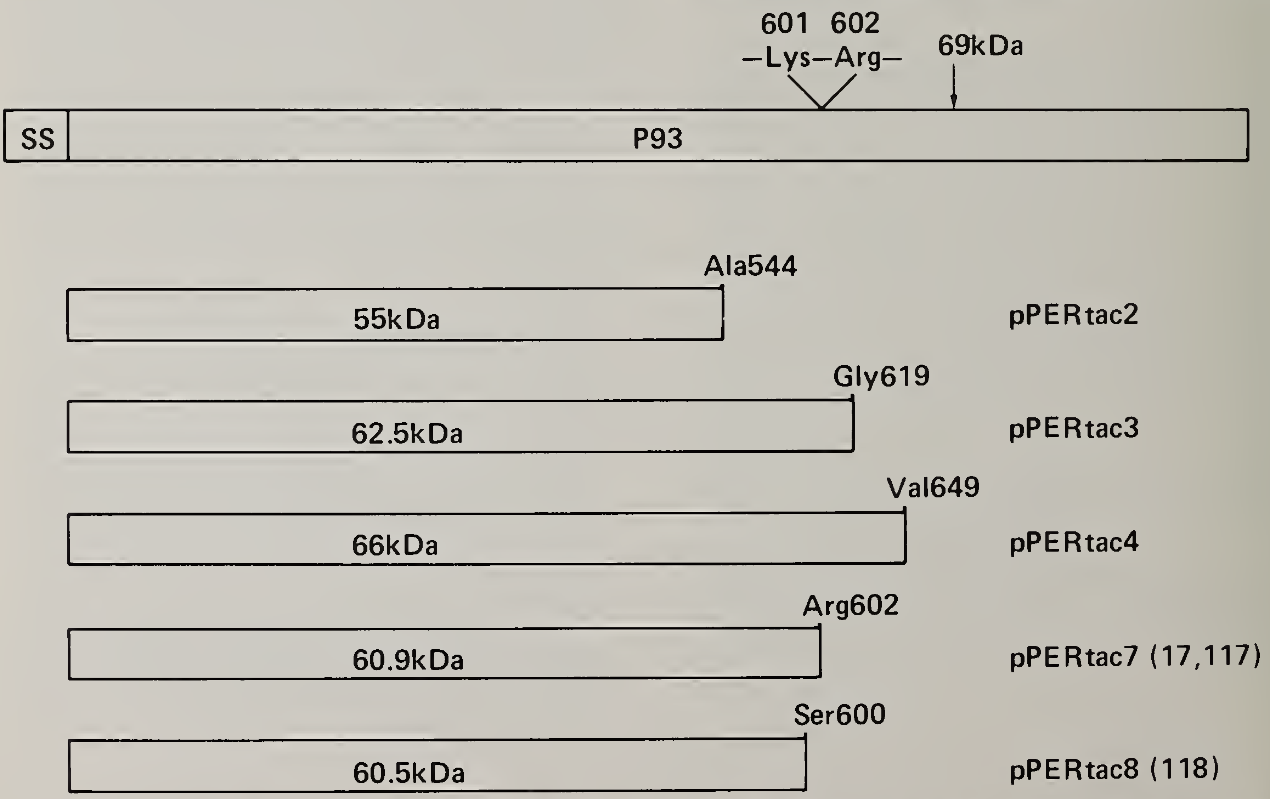

FIG. 1. Structure of P.69 molecules expressed by recombinant plasmids. All proteins begin with Asp-1 and terminate at the residue indicated. The arrow indicates the position of P.69 cleavage which would give a molecule of 69 kDa.

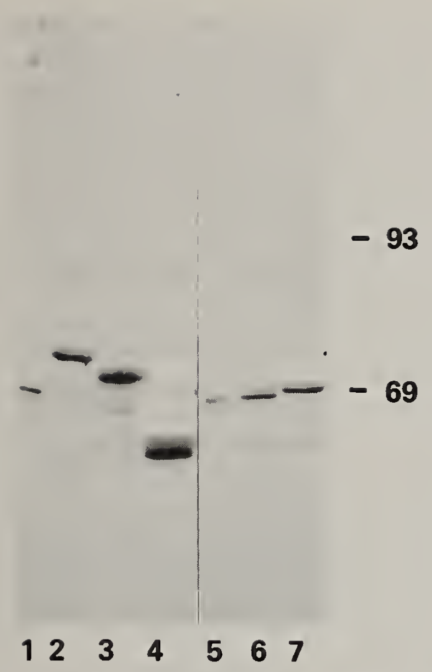

FIG. 2. Expression in *E. coli* of proteins containing P.69 sequences. *E. coli* strains containing the plasmids described in Fig. 1 were grown and induced for the synthesis of P.69 proteins. After SDS-PAGE, the cell lysates were immunoblotted with antibody against P.69. Lanes: 1 and 5, native P.69 pertactin; lane 2, pPERtac4; lane 3, pPERtac3; lane 4, pPERtac2; lane 6, pPERtac8; lane 7, pPERtac7. Size markers are given in kDa.

slower than P.69 (Fig. 2, lanes 1, 2, 3, and 5), suggesting that P.69 is smaller than 62.5 kDa. The carboxy terminus of P.69 was predicted to be upstream of residue 690. Examining this upstream region revealed a pair of basic residues (Lys-Arg) at positions 601 to 602. Since cleavage on the C-terminal side of such residues occurs widely in nature (16), it was resaoned that this might be a likely site for cleavage of P.93. Plasmid pPERtac7 was constructed which terminates translation after Arg-602; this plasmid expressed a protein of 60.9 kDa which migrated close to, but slightly behind, the major component of native *B. pertussis* P.69 (Fig. 2, lane 7). An additional plasmid, pPERtac8, was constructed which terminates expression after Ser-600. This plasmid expressed a 60.5-kDa protein which migrated with the major band of P.69 (Fig. 2, lane 6). Using carboxy peptidase mapping of P.69 purified from *B. pertussis* (Carine Capiau, personal communication, see also this symposium procedings) and by electron spray spectroscopy using native and recombinant P.69 (data not shown), the C-terminus amino acid has been identified as Asn-597.

High-level expression of P.69 in *E. coli*. The plasmid described above expressed the P.69 proteins at about 1% of total *E. coli* cell protein, a much lower percentage than expected from the powerful *E. coli tac* promoter (1). A poor ribosome-binding site on the expression vector used for this study may have been the factor limiting P.69 expression. Experiments with other expression plasmids using a two-cistron system described previously (7) confirmed this finding (Makoff et al., Biotechnology, in press). The DNA sequence immediately around the ribosome-binding was changed by cloning mixed oligonucleotides into pPERtac8 which could result in altered DNA sequence in this region. Transformants were analysed for expression in *E. coli*, and 49 were found which expressed P.69 at a higher level than pPERtac8. Seven transformants produced P.69 at >20% total cell protein; the DNA sequence around the ribosome-binding site of these plasmids was determined. One of the high expressing plasmids, pPERtac36, had two base changes in this region. These changes altered only the DNA sequence in the region involved in ribosome binding; they had no effect on the amino acid sequence of the translated gene product. Plasmid pPERtac36 was therefore chosen for further study.

Purification of recombinant P.69. In contrast with *E. coli* cultures containing pPERtac8, induced cells containing pPERtac36 grew well and expressed reproducibly high levels of P.69 (Fig. 3, lane 1). When these cultures of pPERtac36 were lysed, almost all of the recombinant P.69 was insoluble and found in the pellet fraction (Fig. 1, lanes 2 and 3). P.69 was extracted from *E. coli* (pPERtac36), by French Press lysis of the cells, followed by centrifugation of the lysate to pellet the insoluble inclusion bodies which contained the P.69. The insoluble pellet was washed in Triton X-100 buffer (lane 4) and solubilized it in 6 M GuHCl. The guanidinium was removed by gradual dilution and dialysis, and the P.69 recovered as a fully soluble protein that was over 80% pure as judged by SDS-PAGE (Fig. 3, lane 7). The P.69 was further purified by ion-exchange chromatography using a Mono Q column (data not shown). This simple purification method yielded 80 mg P.69 protein per liter of culture OD 3.0.

CD spectroscopy of recombinant P.69. Circular dichroism (CD) spectroscopy can assess a protein's gross conformational structure, as previously reviewed (13). Using this method, we analyzed and compared the CD spectra of *E. coli*- and *B. pertussis*-derived P.69. Both proteins gave an indistinguishable spectra showing the

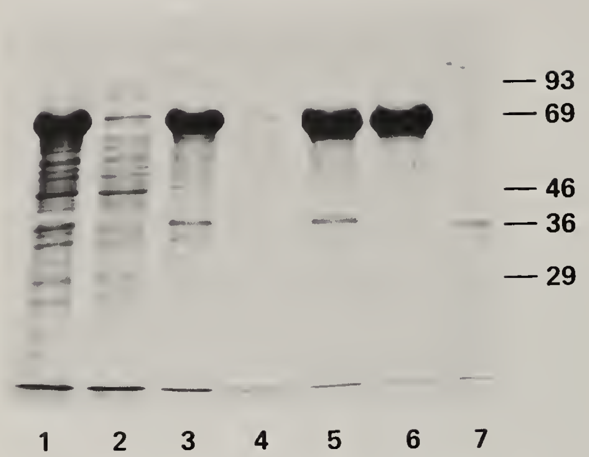

FIG. 3. Purification of *E. coli* P.69 from pPERtac36. Lane 1, whole-cell extract; lane 2, first supernatant fraction; lane 3, first pellet fraction; lane 4, Triton X-100 wash supernatant fraction; lane 5, fraction after solubilization in GuHCl and dialysis; lane 6, supernatant fraction after dialysis; lane 7, pellet fraction after dialysis. Size markers are given in kDa.

TABLE 1. Kendrick test of P.69 samples with pertussis toxoid

Sample[a]	Dose[b]	Survivors[c]
Toxoid 5 μg	neat	10/18
	1/4	2/18
	1/16	0/18
	1/64	1/18
Toxoid 5 μg and P.69 (native) 20 μg	neat	14/18
	1/4	8/18
	1/16	0/18
	1/64	0/18
Toxoid 5 μg and P.69 (*E. coli*) 20 μg	neat	17/18
	1/4	8/18
	1/16	1/18
	1/64	0/18
Reference vaccine (whole-cell 66/84)	neat	15/18
	1/4	7/18
	1/16	1/18
	1/64	1/18

[a] Vaccines were made up with 10% alhydrogel.

[b] A dosage of 0.5 ml was given subcutaneously to NIH(S) mice.

[c] Survivors were counted 14 days after challenge with *B. pertussis* 18323 as described in text.

molecules to be composed primarily of antiparallel β-pleated sheet with very little α-helix (data not shown).

Immunogenicity of recombinant P.69 in Kendrick test. The intracerebral challenge test measures the potency of whole-cell pertussis vaccine, and this laboratory potency has been correlated with clincial efficacy (9). Since P.69 may be used in forthcoming human clinical trials, we examined the ability of purified preparations of P.69 to confer protection to mice in the Kendrick test. Table 1 shows the results of Kendrick tests using pertussis toxoid together with P.69 purified from either *E. coli* or *B. pertussis*. The addition of either recombinant or native P.69 clearly enhances the protection offered by pertussis toxoid to a level equivalent to that of the whole-cell vaccine. Analyzing the slope of the responses shows that the toxoid-P.69 combinations are both linear and parallel to the whole-cell vaccine; pertussis toxoid alone, on the other hand, gives a nonlinear, nonparallel response (data not shown).

DISCUSSION

The family of pertactin proteins (P.68, P.69, and P.70) from *Bordetella* spp. are recognized as important antigens in the pathogenesis of the diseases caused by *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*; their importance as protective antigens is now beginning to be realized (5, 12, 15, 17). We have used recombinant DNA methodology to investigate the structure of P.69 in detail and to synthesize large quantities of this protein in *E. coli*.

Our initial finding was that the size of mature P.69 purified from *B. pertussis* was smaller than the 69 kDa estimated by SDS-PAGE. The C terminus of P.69 was estimated to be Ser-600, based on comparison with a number of recombinant molecules expressing various lengths of P.69. The Ser-600 residue occurs immediately before a Lys-Arg sequence; proteolytic cleavage on the C-terminal side of such di-basic residues occurs widely in nature (16). Thus, it is possible that a protease from *B. pertussis* cleaves the 93-kDa precursor at this Lys-Arg sequence and, perhaps after further cleavage by a carboxy peptidase, Asn-597 is found as the C-terminal residue.

The P.69 molecule contains an unusually high number of proline residues, 7%, compared with an average of 4.6% for sequenced proteins (4). The anomalous behavior of P.69 on SDS-PAGE is likely due to this high proline content, since this results in similar retarded mobility in other proteins (14). Similar behavior has been observed with a recombinant protein expressing the proline-rich domain of P.69 fused to the *E. coli* LT-B gene (M. Lipscombe, personal communication).

Using circular dichroism spectroscopy to compare the structural conformation of *E. coli* P.69 pertactin to *B. pertussis*-derived protein yielded indistinguishable spectra from the proteins, indicating that the expression and purification protocol used for the recombinant P.69 did not cause gross structural changes. The preparation of *E. coli* P.69 pertactin as an insoluble protein followed by solubilization, gradual diluton and dialysis is a highly attractive method for producing large amounts of P.69.

The protective properties of P.69 pertactin by measuring the potency of vaccines in the Kendrick test. A mixture of pertussis toxoid and P.69 (*E. coli*- or *B. pertusssis*-derived) was as protective as the British whole-cell vaccine standard. This finding is encouraging for the development of an acellular pertussis vaccine which can be quality-controlled by the conventional test. Considerable data show that filamentous hemagglutinin (FHA) is also a protective antigen for pertussis (9). Indeed, a recent study (Novotny et al., submitted to J. Infect. Dis.) has shown that a combination of *B. pertussis* P.69 pertactin and FHA can also pass the Kendrick test. Thus, a vaccine consisting of P.69, FHA, and pertussis toxoid in the correct proportions may be the acellular vaccine of choice. Animal tests (e.g., the intracerebral challenge test and the aerosol challenge model) can be used to determine these optimum proportions and establish appropriate doses. In contrast to the limited availability of native P.69, the large yields of recombinant P.69 pertactin allow researchers to use as much of this antigen as necessary. As the availability of this antigen is not limited by growth of *B. pertussis.*

ACKNOWLEDGMENTS

We would like to thank Dr. C. Capiau for supplying toxoided pertussis toxin and *B. pertussis*-derived P.69 for use in the Kendrick tests.

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DISCUSSION

DR. STIBITZ: The increase in expression that you achieved, was that due to disruption of pairing with the ribosome binding site?

DR. FAIRWEATHER: Yes.

DR. STIBITZ: How efficient was your solublization procedure?

DR. FAIRWEATHER: It was very, very efficient. There was virtually none in the pellet. I should perhaps mention that we also used urea to resolublize and while the urea did resolublize it, it also resulted in degradation of the P.69 so in our hands for this particular protein, guanidinium was better.

Autoregulation of the Expression of the Gene for Pertussis Toxin S1 Subunit in *Escherichia coli*

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Pertussis toxin (PT) is a major virulence factor produced by *Bordetella pertussis,* the causative agent of whooping cough. It is a hexameric polypeptide composed of five different subunits termed S1 through S5; S4 is present in two copies. The operon encoding these five proteins has been cloned and sequenced (12, 16). Attempts to express the whole operon in *Escherichia coli* have been unsuccessful, but individual subunits have been produced in this bacterium using various heterologous promoters. The S1 subunit has not been synthesized at very high levels as a mature protein with or without a short fusion peptide. Except in the work of Cieplak et al (7), its detection in recombinant *E. coli* extracts usually requires immunoblotting procedures (3, 5, 13).

The results presented here suggest that the low level of expression of the mature S1 subunit gene is the consequence of transcriptional down-regulation that is effected in an autoregulating fashion by the mature S1 protein.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from New England BioLabs, Inc.; Boehringer Mannheim Biochemicals; or Bethesda Research Laboratories, Inc. T4 DNA ligase was from New England Biolabs. T4 polynucleotide kinase, the large fragment of DNA polymerase I, and isopropyl-β-D-galactoside (IPTG) were from Bethesda Research Laboratories. Calf intestine alkaline phosphatase was from Boehringer Mannheim. Monoclonal anti-S1 antibody 3F10 has been described elsewhere (19). Rabbit anti-mouse immunoglobulin G was purchased from Cappel Laboratories. Nitrocellulose and nylon (GeneScreen Plus) membranes were from Bio-Rad Laboratories and Du Pont Co., respectively. [γ-^{32}P]ATP (3,000 Ci/mmol) and [α-^{35}S]dATP (500 Ci/mmol) were purchased from New England Nuclear Corp. MilliGen kindly provided the oligonucleotides used in pTXS13/3 subcloning.

Bacterial strains and plasmids. *E. coli* strains JM101 and JM109 and plasmid pUC8 have been described elsewhere (22), as have the *E. coli* strain HMS273(pGP1-2) and the plasmid pT7-7 (kindly provided by S. Tabor) (20) and plasmids pTXS13 and pTXS13/R9K (13, 10). Plasmid pTXS13B was constructed by ligation of the 1.69-kb Xmn I fragment of pTXS13 with the 1.85-kb Xmn I/Sma I fragment of pUC8. Plasmid pTXS13/3 was constructed by ligation of the 2.67-kb Acc I/Hind III and the 0.79 Xmn I/Hind III fragments of pTXS13 using a synthetic linker inserted between the Acc I and Xmn I sites. This linker, carrying three silent mutations (shown in boldface type) was prepared by annealing the following phosphorylated oligonucleotides: ATACCGCTATGACTC/CCGCCC**A**CC**T**GAG**G**ATGTTTTCCAGAACG and CGTTCTGGAAAACATCCTCAGGTGGGCGGGAGTCATAGCGTG. To construct pTXS13 of, pTXS13 was digested with BamH I which cuts at the 5′ junction of the insert with the vector. The BamH I protruding ends were filled in with the Klenow fragment of the DNA polymerase I and then ligated. Before its transformation into JM101, the ligation mixture was digested with BamH I to linearize any plasmids still containing this restriction site. The transformants were screened for the absence of the BamH I site in the recombinant plasmid, and one colony was selected. DNA sequencing analysis of the plasmid carried by these cells showed that the fill-in reaction was partial. The resulting sequence was . . . CCGGGG**AT**ATCCTCCCG . . . (the vector sequence is underlined, and the added nucleotides are boldfaced). To create pTK13, an Ssp I/Sca I fragment of

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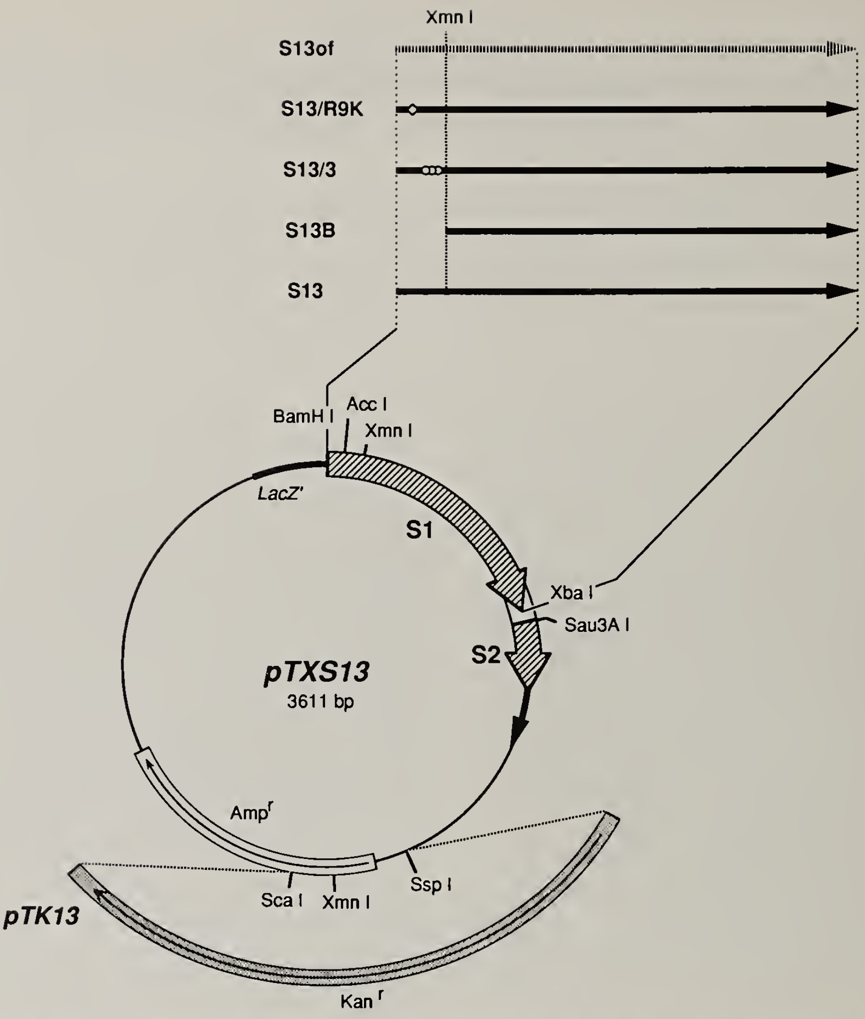

FIG. 1. Restriction map of pTXS13 and map of the S1 analog genes. The upper part represents the structure of the S1 analog genes. The circles in S13/3 and the diamond in S13/R9K indicate the position of the mutations; the dotted line in S13of indicates that the gene is out of frame. The lower part shows a partial restriction map of pTXS13. The shaded area at the bottom denotes the difference between pTXS13 and pTK13.

pTXS13, which contains the 5′ third of the ampicillin resistance gene and its ribosome-binding site, was replaced by the 1.7-kb Hinc II fragment of pUC4K (Pharmacia, Inc.), containing the kanamycin resistance gene of Tn903 (21). The transformants were grown on plates containing kanamycin and screened for the presence of the kanamycin resistance gene in the same orientation as the original ampicillin resistance gene. The parent plasmid pTXS13 and its derivatives are illustrated in Fig. 1.

DNA manipulations. Most procedures were carried out using standard methods (2). When applicable, the vectors involved in cloning experiments were dephosphorylated with calf intestine alkaline phosphatase prior to ligation. DNA fragments isolated by agarose gel electrophoresis were either recovered by electroelution onto NA45 DEAE-membranes (Schleicher & Schuell, Inc.) or used directly in the presence of the agarose after melting the excised block. In the latter case, SeaPlaque agarose (FMC BioProducts) was used exclusively. Transformations were performed according to the protocols described by Hanahan (8) or Chung et al. (6).

RNA preparation and Northern analysis. *E. coli* cells containing the plasmid(s) of interest were grown at 37°C in LB medium containing the appropriate antibiotics. When the optical density at 600 nm reached a value of 0.3 to 0.4, IPTG was added to the medium at a final concentration of 1 mM and the cells were allowed to grow for 2 more hours or overnight. The cells were harvested by centrifugation and their RNA fractions prepared as described by Anderson et al. (1). Northern analysis was performed using the glyoxal/dimethyl sulfoxyde denaturation method (2). The RNA was subsequently transferred in 20 x SSPE onto a nylon membrane by negative pressure using a VacuGene apparatus from Pharmacia-LKB. Membrane treatment before and after transfer and during prehybridization and hybridization was performed according to manufacturer recommendations. Hybridization was conducted overnight at 37°C with ^{32}P-labeled "M13 Probe Primer" (Bethesda Research Laboratories). The membranes were then washed three times for 10 min. in 6 x SSPE at 20°C and submitted to autoradiography using Kodak X-Omat AR5 films and Cronex Lightning-Plus intensifying screens.

Other analytical procedures. DNA sequencing was performed by the dideoxy chain-termination method (18) with Sequenase (United States Biochemical Corp.) according to the manufacturer's recommendations. DNA computer analysis was done using Microgenie (Beckman Instruments, Inc.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins and their transfer on nitrocellulose membranes were carried out according to Laemmli (9) and Burnette (4), respectively. Quantitative densitometric scanning of SDS-polyacrylamide gels and immunoblots were carried out using a Pharmacia-LKB Ultroscan XL laser densitometer.

RESULTS

Expression of a 5′ truncated S1 gene. A 5′ truncated gene of PT S1 subunit was subcloned from the parent plasmid pTXS13 (13) into pUC8 as described above. The resulting plasmid, termed pTXS13B, encoded a protein (S13B) composed of the S1 subunit without the 22 amino-terminal amino acids of the mature protein, preceded by 8 amino acids translated from the 5′ end of the *lacZ* gene and the multiple cloning site (Fig. 1). After transformation of pTXS13B into JM101, the expression of the S13 and S13B genes was induced with IPTG, and protein production was monitored using SDS-PAGE and immunoblotting. Fig. 2 shows that the truncated version of S1 (S13B) was produced at an increased level when compared to the full-length protein (S13). S13B was readily visible after the gel was stained with Coomassie blue (Fig. 2a) while detection of S13 required immunoblotting. Surprisingly, S13B was also reactive with MAb 1B7, although it lacked a portion of the epitope recognized by this MAb (7).

Depending on the growth conditions and the duration of induction, S13B production was estimated to be increased by a factor of 5 to 50 over that of the full-length S13. This reproducible increase of production contrasts with results obtained in other studies where no significant difference was observed between the synthesis of full-length and various truncated analogs of S1 in *E. coli* (7, 15). However, in these studies the S1 analogs were produced using different expression systems. To determine if this difference in synthesis was peculiar to the present study's plasmid-borne expression system, the S13 gene and its truncated form S13B were subcloned in the expression plasmid pT7-7 under control of the T7 polymerase promoter (20). We subsequently observed a similar difference in synthesis with this vector (not shown). These observations indicate that the increased level of synthesis of S13B is peculiar to neither the cloning vector (pUC or pT7-7) nor RNA polymerase (from *E. coli* or T7 bacteriophage) involved, and appears

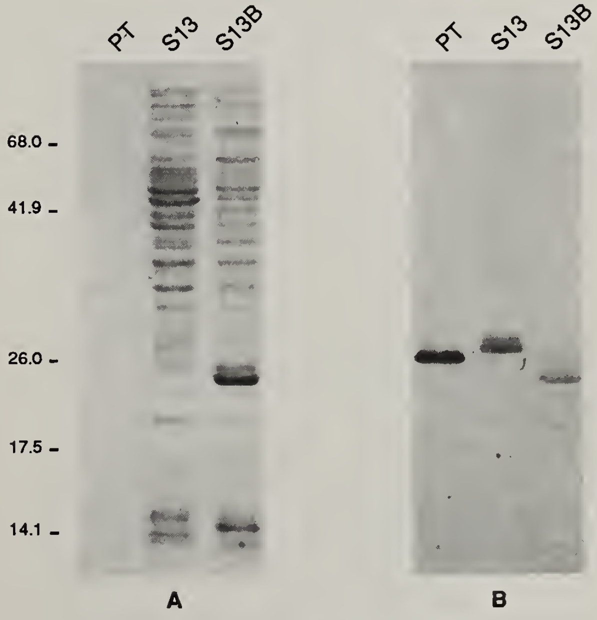

FIG. 2. SDS-PAGE and Western blot of pUC18, pTXS13, and pTXS13B. Following an overnight induction in 1 mM IPTG, cells containing the various pUC-derived plasmids were harvested. They were resuspended in SDS-PAGE sample buffer at a final concentration of 20 OD units per ml and boiled. Ten μl of this solution was submitted to SDS-PAGE. Gels were either stained with Coomassie blue (A) or electroblotted onto nitrocellulose. In the latter case, the S1 subunit analogs were detected using MAb 1B7 (B). The M_r markers, in kDa, are shown on the left of the figure.

to be directly linked to the absence of the N-terminus of the protein and/or the 5′ end of the S1 subunit gene.

These results are in apparent disagreement with those of Barbieri et al. (3) who have expressed a truncated S1 subunit identical to S13B using the same vector (pUC8). Their plasmid construction, however, was slightly different from that used here, since the sequence downstream of the Xba I site which contains the translational stop codon of S1 (Fig. 1) was absent. Analyzing the production of S13B by such a construct revealed that the synthesis was depressed compared to that using the plasmid with the S2 sequence 3′ to the Xba I site (not shown). No difference was observed in S13 production in the presence or absence of the sequences downstream of the Xba I site. Further analysis indicated that the removal of the sequence downstream of the Sau3A I site (Fig. 1) did not affect S13B expression, indicating that removal of the intervening sequence between the genes for S1 and S2 is responsible for the decrease of S13B expression. Interestingly, short direct and inverted repeats are present in this area.

Computer analysis of the 5′ end of the mature S1 gene indicated the presence of a theoretical stem-loop structure with a free energy of -20 KCal. Such structures can reduce the efficiency of the translation when they are located near the start codon (14, 17). This potential secondary structure is absent in pTXS13B, since the 5′ part of the stem has been removed by the truncation. This absence might explain the increased synthesis of S13B. To investigate this hypothesis, silent mutations were introduced at the 5′ end of the S1 gene to reduce the complementarity in the stem while leaving the protein sequence intact. The mutation of three nucleotides (pTXS13/3) in this area resulted in a calculated increase of the free energy of the secondary structure to about -12 KCal, at which level the effect on the translation is expected to be much weaker. Upon induction with IPTG, JM101(pTXS13/3) produces S13 at a level equivalent to that of JM101 containing pTXS13 (not shown). This result indicates either that this theoretical secondary structure has no influence on the translation of S13 or that the stem-loop structure does not occur in vivo.

The expression of S13 is down-regulated at the transcriptional level. Experiments were designed to analyze the effects of S1 gene truncation on its transcription. Using Northern analysis, we compared the transcription level of the genes encoding l*acZ*′, S13, S13B, and S13of in *E. coli.* The mRNAs synthesized from the *lacZ* promoter were detected by hybridization with the M13 Probe Primer (Bethesda Research Laboratories), an oligonucleotide recognizing a sequence overlapping the start codon of the l*acZ*′ gene, upstream of the multiple cloning site. Using this probe, the mRNAs synthesized from all the modified S13 genes inserted in the multiple-cloning site of any pUC derivative could be detected and any possible degradation at the 3′ end of these molecules identified. Fig. 3 shows that the amounts of mRNA synthesized from pUC18, pTXS13B, and pTXS13 of are similar, while JM101(pTXS13) directs the synthesis of a significantly lesser amount of l*acZ*′-derived mRNA. This result indicates that the low level of expression of S13 is, at least partially, the consequence of the inhibition of transcription. The amount of mRNA synthesized from pTXS13of is much greater than from pTXS13, indicating

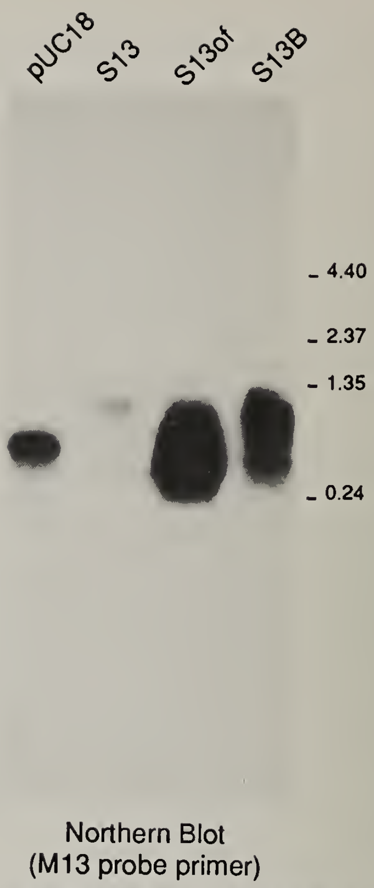

FIG. 3. Northern blot analysis of S1 subunit analogs mRNA. After a 2-hour induction in 1 mM IPTG, cells containing the pUC-derived plasmids were harvested and their RNA fractions prepared. Five µg of total RNA from each strain was submitted to agarose gel electrophoresis and transferred to a nylon membrane. The blot was then probed with ^{32}P-labeled "M13 Probe Primer" to visualize the mRNA transcribed from the l*acZ* and the S1 analog genes. The size markers, in kb, are shown on the right of the figure.

that down regulation did not occur in this case. Since (i) no S13 analog is synthesized from pTXS13of and (ii) the S13 gene sequence is intact in this plasmid, the transcription inhibition therefore requires the presence of the gene product and is not the direct result of secondary structures of the DNA and/or the RNA. Finally, the ADP-ribosyltransferase activity of S13 is not required for the down regulation to occur: the pTXS13/R9K insert, which produces a mutant protein with significantly reduced ADP-ribosyltransferase activity (0.08% of wild-type) (10), is transcribed at the same low level as the insert of pTXS13 (not shown).

DISCUSSION

The results presented here indicate that, in *E. coli*, the expression of the S1 subunit gene of PT is autoregulated, i.e., the product of the S1 gene appears to repress its own transcription. The interaction exhibits the following characteristics: (i) it results in a significant decrease in the amount of S1 mRNA synthesized; (ii) it is independent of the type of plasmid that carries the S1 gene and independent of the RNA polymerase responsible for the transcription; and (iii) the interaction is not dependent on the ADP-ribosyltransferase activity of the S1 subunit. The precise molecular mechanism of this interaction is not clear. DNA binding experiments as well as in vitro transcription assays have so far failed to demonstrate any physical interaction between S13 and its DNA and/or RNA. However, the binding conditions used in these assays may not have been optimal.

Other observations support the finding of autoregulation of S1 subunit expression. It was recently discovered (11) that some commercially available pUC18 plasmids carry a single-base deletion upstream of the multiple-cloning site. This deletion, which results in a shift of reading frame, results in *lacZ′* product synthesis that is 20 to 50 times lower than that derived from the wild-type plasmid (11). Originally the mature S1 gene was subcloned in a vector which contained this deletion and its expression level was found to be rather low. The subcloning of this gene in a wild-type pUC18 plasmid increased the expression of the S1 subunit only by a factor of 2. However, when the 5′ truncated version of S1 gene was subcloned in both wild-type and mutant pUC plasmid, the difference in expression between the two recombinant S13B plasmids was similar to that observed with *lacZ′*, which suggested that the amount of full-length S1 subunit expressed was controlled by some intrinsic regulatory process (unpublished results).

The observations of Burnette et al. and Cieplak et al. concerning the expression of S1 subunit genes in *E. coli* are also relevant to the current discussion. Burnette et al. (5) found that a construct encoding the mature S1 sequence with an amino-terminal methionine resulted in the production of virtually undetectable (trace) amounts of S1. In a subsequent study, however, Cieplak et al. (7), using the same expression vector, found that substituting the first two aspartic acid residues of the S1 subunit with a methionine-valine dipeptide resulted in the production of considerable amounts of S1 protein (10 to 20% of total cell protein). Coupled with the findings of the present study, these observations suggest that the amino-terminal region, including the two aspartic acid residues and/or the 5′ end of the structural gene, are essential to the autoregulation phenomenon.

Further characterization of this transcriptional regulation and its relevance in *B. pertussis* are currently under investigation.

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DISCUSSION

DR. PARKER: Did you look in your northern blots to see if there was any evidence of an anti-sense messenger RNA?

DR. LOBET: No.

DR. ECKHARDT: Did you have a chance to look at some of your mutants to see if they are still autoregulated or not or if there is a correlation between enzymatic function?

DR. LOBET: No, I showed the construct on the slide. I did not give you the reasons. It is not dependent on the enzymatic activity.

PARTICIPANT: A similar kind of regulation has been demonstrated in ribosomal protein in *E. coli* S4 and three or four proteins which are on the same operon. That has been demonstrated by protein DNA binding. I was wondering if you have done a similar experiment on DNA and S13 gene product binding, foot printing to demonstrate that possible binding.

DR. LOBET: We are currently trying to do this kind of experiment.

DR. LOCHT: Since you are working with a multicopy plasmid, an alternative explanation would be that there is a regulation of plasmid replication rather than transcriptional. Did you check for that?

DR. LOBET: Yes, we did actually and you have no change there.

SESSION FIVE

Pertussis Epidemiology, Diagnosis, and Clinical Studies

Co-chairpersons:

Bruce D. Meade
C. H. Wirsing Von König

Introduction

BRUCE D. MEADE

Approximately two and a half years ago, many of us met in this room to hear the preliminary results from the efficacy trial in Sweden. Many walked away from that meeting somewhat disappointed. The disappointment, however, was not from the conduct of the trial for I think the trial will certainly serve as a model for any future trials on pertussis vaccines. I think the disappointment came from the fact that there were many unanswered questions, some unanswered questions regarding safety, some regarding efficacy and certainly uncertainty of laboratory correlates of clinical protection. Since that meeting, many individuals worked very hard to further understand and evaluate the trial.

Our next three speakers are among those who have devoted considerable time and effort to that endeavor. This afternoon, they will present their current understandings of the trial in Sweden. In the fourth presentation, David Miller from the UK will present preliminary results from a safety and immunogenicity trial recently conducted in the United Kingdom. This actually was an invited paper for we felt that this would be very valuable information for those of us who are currently involved in similar trials. The next paper will deal with outstanding issues related to the clinical evaluation of acellular vaccines. The last four papers in the session are concerned with the epidemiology and diagnosis of pertussis.

Evaluation of Laboratory Methods Used for the Diagnosis of Pertussis Infection and Disease in the Swedish Efficacy Trial

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Laboratory confirmation of pertussis infection is crucial for correct estimates of pertussis vaccine efficacy (4). To reduce the risk of false positive diagnosis, culture confirmation was chosen as the main endpoint in a Swedish placebo-controlled double-blind efficacy trial of two acellular vaccines. The trial entailed active surveillance of nearly 4,000 children during 1986 to 1987. Vaccine efficacy estimates against culture-confirmed pertussis were 54% for a monocomponent vaccine, JNIH-7, which contained formaldehyde-inactivated pertussis toxin (PT) and 69% for a two-component vaccine, JNIH-6, which contained filamentous hemagglutinin (FHA) as an additional protective antigen (1).

The aims of this research were to (i) use sensitive serological criteria to refine the original estimates of vaccine efficacies and explore how vaccine efficacies vary with clinical severity, (ii) explore the effect of vaccination on bacterial isolation rates in serologically confirmed cases, and (iii) compare the sensitivities of the serological assays in vaccinated and nonvaccinated study children with culture-verified pertussis infection.

(This report is based on reference 5 and H. Hallander, J. Storsaeter, and R. Möllby, submitted for publication.)

MATERIALS AND METHODS

Study children received two doses of either JNIH-6 vaccine ($n = 1,389$), JNIH-7 vaccine ($n = 1406$), or placebo ($n = 929$). Clinical data were collected and laboratory analyses were performed blindly during the trial. Pernasal swabs and blood samples were taken if (i) a child had coughed for 7 days or more, (ii) whooping cough was suspected, or (iii) the child had been exposed to culture-verified *Bordetella pertussis* or *B. parapertussis*. Acute blood samples were taken within 14 days of onset of cough, or as soon as possible after exposure to pertussis; convalescent samples were taken at least 6 weeks later.

Specimens for culture were handled and processed at nine bacteriological laboratories using strictly standardized routines. Pernasal swabs were inoculated in the home on selective charcoal agar plates containing 10% defibrinated horse blood and 40 mg/liter of cephalexin. The swabs were then inserted in tubes with a half-strength of the same agar for enrichment. Subcultures from the tubes were performed after 72 h.

The plates were observed regularly from the third to the seventh day after inoculation. Suspected colonies were identified by slide agglutination using specific antisera against *B. pertussis* and *B. parapertussis*. Final biochemical verification was performed at the National Bacteriological Laboratory in Stockholm.

All diagnostic sera were analyzed for antibodies against PT by the Chinese hamster ovary (CHO) cell test, described elsewhere (3).

A carefully standardized system for enzyme-linked immunosorbent assay (ELISA) testing with new reference sera and unit calculations was developed (2). In this system, sera were assessed in eight twofold dilutions, starting at 1/60 using purified PT and FHA from Biken, Japan, as an antigen. A TECAN sampler 505 was used for titration. Paired sera from the patient were always run on the same plate together with a reference serum (Hillman, NBL, containing 1,000 PT units and 1,000 FHA units/ml) and a control serum (IgG42) and incubated for 2 h. Retesting was done in cases of evident technical disorders, e.g., when the background was high or when the control was outside predetermined limits. A computer-modified parallel line bioassay was used to

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* Presenting author.

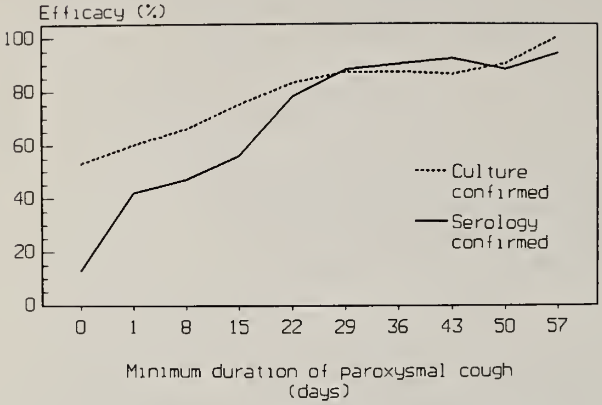

FIG. 1. Vaccine efficacy for the monocomponent vaccine JINH-7 against pertussis infection confirmed either by ELISA titer rise against FHA or by culture, related to duration of paroxysmal cough (reference 5; with permission from Butterworth-Heinemann Ltd., publishers).

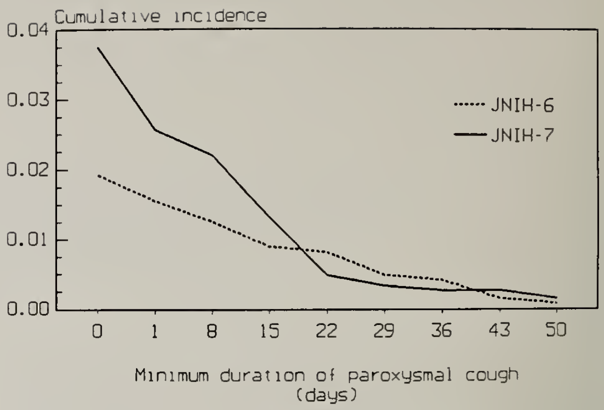

FIG. 2. Cumulative incidence of pertussis infection diagnosed by titer rise in the CHO cell test or by ELISA titer rise against PT for JNIH-7 recipients and recipients of the two-component vaccine JNIH-6, related to duration of paroxysmal cough (reference 5; with permission from Butterworth-Heinemann Ltd., publishers).

express the results in units; the method was based on the logarithm of optical density values between 0.1 and 2.0, read at 405 nm, and plotted against dilution steps. When the test serum line is assumed to be parallel to the reference serum line, the distance in dilution steps between the straight lines for the test and reference sera indicates the relation between the number of units in the two sera. For samples without detectable antibodies, the results were set to the lower detection limit of the assay. These lower detection limits were 2 units for IgG ELISA against FHA, 5 units for IgA ELISA against FHA and IgG ELISA against PT, and 10 units for IgA ELISA against PT.

The diagnostic serological criteria used in the secondary analyses were based on intra-assay variability and aimed at high sensitivity and acceptable specificity. An IgG ELISA titer rise against PT or FHA of 50% or more was regarded as significant, whereas a titer rise of 100% or more was required for IgA ELISA against PT or FHA.

RESULTS AND DISCUSSION

Because the specificity of the serological assays was uncertain, very strict serological criteria were used in the original efficacy analysis, and few culture-negative serologically-positive cases were identified (1). More sensitive serological criteria allowed serological confirmation of additional cases in the trial cohort.

Serological comparison of JNIH-7 with placebo. A secondary analysis of efficacy has been performed; this analysis was restricted to the assays in which pre-exposure titers were expected to be similar between groups. The JNIH-7 group, like the placebo group, did not have pre-exposure titers against FHA. Fifty-five JNIH-7 recipients and 42 placebo recipients were identified during the trial with ELISA titer rise against FHA. For 22 of 55 culture-negative children in the JNIH-7 group, there were no concomitant titer rises against PT in the ELISA assays or in the CHO cell test. Of those 22 children, 15 had documented recent exposure to pertussis in family or day care; 2 more had documented possible exposure in the neighborhood. In the placebo group there were six culture-negative children with titer rises only in FHA, and none of them had recent documented exposure to pertussis.

The bacterial isolation rates were higher in the placebo group than in the JNIH-7 group. Thirty out of 42 placebo recipients were confirmed by culture (71%), compared to 17 out of 55 JNIH-7 recipients (31%) ($P = 0.0002$).

The observed difference in bacterial isolation rates is unlikely to be the result of false positive serological diagnosis. The overall efficacy estimate against pertussis infection was lower when using diagnoses based on FHA serology than when using diagnoses based on culture (Fig. 1). There was no indication of protection against pertussis infection per se, but the protective efficacy increased with increasing severity of the clinical case definitions (Fig. 1). Using a case definition that required more than 3 weeks of spasmodic coughing plus

TABLE 1. Proportion of significant titer rises in 71 study children with culture-verified pertussis infection and diagnostic serology

Serological assay	JNIH-6 $n = 16$ No. (%)	JNIH-7 $n = 21$ No. (%)	Placebo $n = 34$ No. (%)
CHO cell neutralization	6 (38)	7 (33)	29 (85)
ELISA PT IgG	6 (38)	11 (52)	34 (100)
ELISA PT IgA	0 (0)	2 (10)	19[b] (59)
ELISA FHA IgG	16 (100)	17 (81)	30 (88)
ELISA FHA IgA	10[a] (67)	8 (38)	17[b] (53)

[a] n=15.
[b] n=32.

ELISA titer rise against FHA yielded an efficacy estimate of 78% (95% confidence interval [CI] 56, 89).

Serological comparison of JNIH-6 with JNIH-7. All vaccinated children had prior exposure to formaldehyde-treated PT. The researchers identified 25 JNIH-6 recipients and 50 JNIH-7 recipients with diagnostic ELISA titer rise against PT, or a rise in the CHO cell assay, after the day of the post-vaccination blood sample. Fig. 2 suggests that the two-component vaccine JNIH-6 provided more protection against pertussis infection than the monocomponent vaccine JNIH-7. The difference in cumulative incidence rates between the two groups was statistically significant only when four asymptomatic JNIH-7 recipients with titer rises were included as cases of pertussis (RR, 1.95; 95% CI 1.22, 3.14). More asymptomatic and mild cases with serological evidence of pertussis were found in the JNIH-7 group, both proportionally and in absolute numbers. Thus, 44 of 50 cases (88%) in the JNIH-7 group had paroxysmal cough lasting less than 22 days, compared to 15 out of 25 cases (60%) in the JNIH-6 group ($P = 0.013$). No difference was found between the two vaccine groups when the case definition required more than 3 weeks of spasmodic coughing plus ELISA titer rise against PT or in the CHO cell test (RR, 0.59; 95% CI 0.21, 1.63). There were no significant differences in bacterial isolation rates between the two groups.

Diagnostic serology in children with culture-verified *B. pertussis* infection. Table 1 shows the proportion of significant titer rises in the different serological assays used during the efficacy trial. Data are available for 71 study children with culture-verified infection and diagnostic serology.

For all assays that measured antibody titer rises against PT, there was a statistically significant differential sensitivity between vaccinated and unvaccinated children. A significant IgG ELISA titer rise against PT was obtained for all 34 placebo recipients (100%), but only for 17 of 37 vaccinated children (46%, $P < 0.001$). The CHO cell was less sensitive and could diagnose 29 of 34 (85%) placebo recipients, compared to 13 of 37 (35%) vaccine recipients ($P < 0.001$). ELISA IgA against PT demonstrated very low diagnostic sensitivity. Only 2 of 37 (5%) of the vaccinated children had a significant titer rise in this assay, compared to 19 of 32 (59%) in the placebo group ($P < 0.001$).

Overall, the most sensitive test was IgG ELISA against FHA. This test was sensitive even for JNIH-6 recipients who had been primed with FHA. The assay could diagnose 30 of 34 placebo recipients (88%), compared to 17 of 21 JNIH-7 recipients (81%) and 16 of 16 (100%) JNIH-6 recipients. However, this assay had problems

TABLE 2. Mean difference between log convalescent and log acute titers ± SD and mean log convalescent titers ± SD for 107 children with culture-verified pertussis after the day of the first trial injection

Assay	JNIH-6[a]		JNIH-7[b]		Placebo[c]	
	Titer diff	Conv titer	Titer diff	Conv titer	Titer diff	Conv titer
IgG PT	0.47 ± 0.56	2.03 ± 0.38	0.24 ± 0.44	2.12 ± 0.46	1.48 ± 0.64	2.29 ± 0.53
IgA PT	0.05 ± 0.49	1.29 ± 0.56	0.07 ± 0.52	1.46 ± 0.57	0.57 ± 0.66	1.73 ± 0.63
IgG FHA	0.79 ± 0.36	1.94 ± 0.38	0.76 ± 0.44	1.11 ± 0.45	0.95 ± 0.57	1.34 ± 0.60
IgA FHA	0.56 ± 0.65	1.54 ± 0.65	0.25 ± 0.30	1.01 ± 0.42	0.43 ± 0.43	1.16 ± 0.45

[a] $n = 25$.
[b] $n = 33$.
[c] $n = 49$.

with cross-reactivity with other *Bordetella* species. Children with parapertussis infections had antibody titer rises against FHA of the same magnitude as pertussis cases (data not shown).

Sixty-nine of 71 (97%) of the children could be diagnosed with the combined use of IgG ELISA against PT and/or IgG ELISA against FHA.

Mean ELISA titer rises and convalescent titers in children with culture-verified pertussis infection. Table 2 summarizes serological data from 107 study children with culture-verified pertussis infection after the day of the first injection in the trial. A separate analysis, restricted to cases with diagnostic serology according to protocol (i.e., the acute blood sample taken after the day of the post-vaccination sample, which was 2 months after the second injection in the trial), gave similar results (data not shown). The median interval from onset of cough to the day of the acute blood sample was 7 to 8 days in all three study groups.

The table shows that the placebo group had a significantly higher mean titer rise in paired sera measured by IgG ELISA against PT than the two vaccine groups. The differences in mean titer rises of the placebo and the two vaccines were 1.24 for JNIH-7 (95% CI 1.0, 1.5) and 1.01 for JNIH-6 (95% CI 0.7, 1.3). The mean log convalescent IgG ELISA titer against PT was marginally statistically significant lower in the JNIH-6 group compared to the placebo group.

The results imply that serological diagnoses based on titer rises against PT in paired sera may be biased when comparing vaccinated and unvaccinated children.

The serological responses measured by IgG ELISA against FHA demonstrated a different pattern. The JNIH-6 group, which had been immunized with FHA, had higher convalescent titers than the two other groups, whereas titer rises were comparable in all three groups.

The higher diagnostic sensitivity observed for IgG ELISA against FHA than for IgG ELISA against PT may be explained if the serological titer rises against FHA continue for a longer period of time than the titer rises against PT. For primary immunized children, the greater part of the titer rises against PT may take place before the acute blood samples are obtained, even under optimal diagnostic conditions.

Conclusions. The study results confirmed that cultures had low sensitivity and were more likely to diagnose unvaccinated than vaccinated children with pertussis infection. IgG ELISA was the method of choice for measuring antibody response against PT. However, serological diagnosis based on titer rises against PT in paired sera was more likely to diagnose unvaccinated than vaccinated children with culture-verified pertussis infection. Both of these diagnostic biases will tend to produce falsely high estimates of vaccine efficacy in a placebo-controlled trial.

ELISA measurement of antibody titer rises against FHA improved diagnostic sensitivity in all three study groups but reduced diagnostic specificity. Children with *B. parapertussis* infections had titer rises against FHA of the same magnitude as children with culture-verified *B. pertussis* infections.

The use of sensitive serological assays for FHA antibody reduced estimates of vaccine efficacy, especially when all children with serological titer rises — even those with mild and atypical symptoms — were included as cases of pertussis.

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Acellular Pertussis Vaccine Efficacy Determined from Clinical Criteria

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A randomized placebo-controlled double-blind trial of two Japanese acellular pertussis vaccines was conducted in Sweden during 1986-1987 (1). The trial resulted in estimates of vaccine efficacy against all culture-confirmed illness of 69% for a two-component vaccine and 54% for a single-component vaccine. These results were received with some disappointment (6). However, results of post-trial passive surveillance suggest that the active surveillance during the trial probably detected many cases of mild disease that would have gone unrecognized under routine circumstances, producing lower efficacy estimates than would have been obtained otherwise (P. Olin, J. Storsaeter, V. Romanus, Letter to the editor, J. Am. Med. Assoc. 261:560, 1989).

The present study estimated efficacy from the Swedish trial data using various clinical case definitions, compared the estimates with those obtained when culture confirmation was required, and considered the suitability for a future trial of a case definition based solely on clinical criteria.

MATERIALS AND METHODS

The methods of the trial have been presented in more detail elsewhere (1, 3, 9).

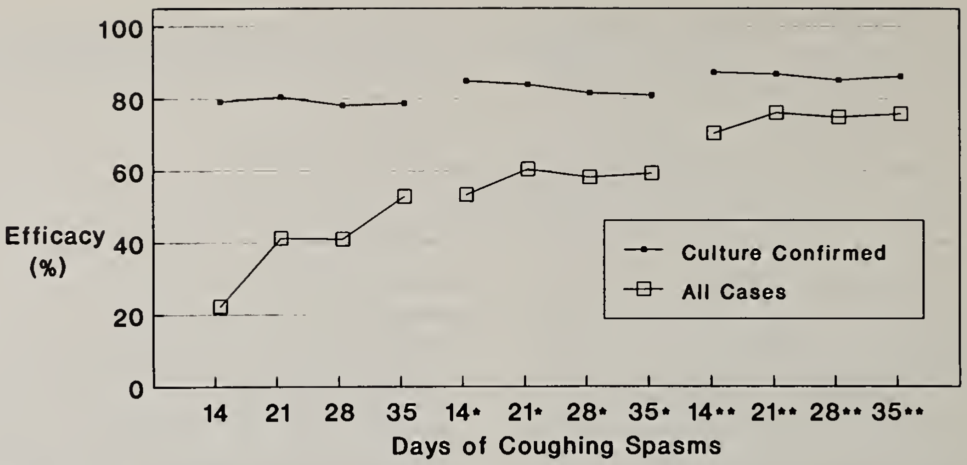
Children aged 6 months to 1 year received two subcutaneous injections of either JNIH-6, a vaccine containing detoxified pertussis toxin (PT) and filamentous hemagglutinin; JNIH-7, a single-component vaccine containing only pertussis toxoid; or placebo. Children were then observed for possible pertussis disease until they either reached the end of the study or had culture-confirmed pertussis. During this 15-month follow-up period which began 30 days after the second injection of vaccine or placebo, cultures and blood samples were taken if the children coughed for at least 7 days or were suspected to have been exposed to pertussis.

The actuarial method was used to estimate pertussis incidence for a variety of case definitions (7). Although some children had more than one recorded episode, a child was counted as a case only once for any particular case definition. All recorded episodes were used in the analysis, however. The standard formula, $1 - p_v / p_u$, where p_v and p_u are observed incidence in vaccinated and unvaccinated children, respectively, was used to estimate vaccine efficacy. Since there was very little loss to follow-up, differences between the efficacies of JNIH-6 and JNIH-7 were tested by treating the ratios of cases to total children as simple binomial proportions and using the chi-square test with continuity correction (2). In the chi-square test, $P < 0.05$ was considered statistically significant, without correction for the number of tests done. (The small loss to follow-up also means that efficacy estimated from the ratio of binomial proportions would be almost the same as that obtained using the actuarial method.)

Vaccine efficacy was estimated using case definitions based on various combinations of the following criteria: duration of coughing spasms (at least 14, 21, 28, or 35 days), whoops, and nine or more coughing spasms on at least one day. This last criterion has been used in the past as an indicator of moderate or severe illness (1, 5). Efficacy was also estimated when culture confirmation was required as part of the definition.

RESULTS

The numbers of children with at least 30 days follow-up after the second injection were 1,385 for JNIH-6, 1,403 for JNIH-7, and 923 for placebo. Figures 1 and 2 show estimates of vaccine efficacy as well as numbers of cases for a variety of clinical case definitions. They give curves of efficacy estimates both for all cases meeting the clinical definition and for culture-confirmed cases only. The graphs on the left in each figure give efficacy estimates for cases with at least 14, 21, 28, and 35 days of coughing spasms, respectively; the graphs in the center give estimates for the same durations of coughing spasms when whoops were added to the case definition; and the graphs on the right give estimates when both whoops and at least nine spasms in a day were required.

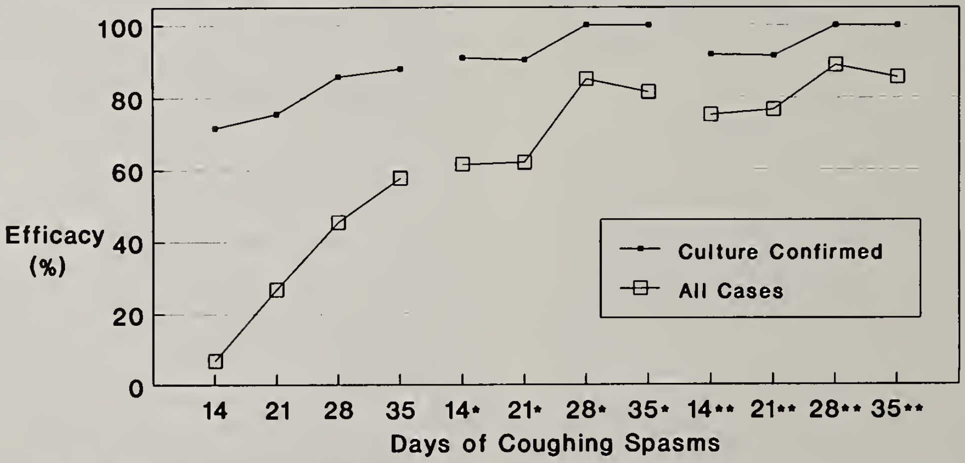


No. of Cases													
Culture	JNIH-6	11	10	10	8	7	7	7	6	5	5	5	4
Conf.	Placebo	37	35	31	26	32	30	26	22	26	27	24	21
All	JNIH-6	176	94	65	36	40	27	22	15	20	13	11	6
Cases	Placebo	151	107	73	54	57	46	35	25	46	36	30	23

* *with whoops*
** *with whoops and >=9 spasms on at least one day*

FIG. 1. Vaccine efficacy, JNIH-6: n = 1,385 (JNIH-6), 923 (placebo).

Estimates of efficacy varied widely with the clinical criteria. Efficacy generally increased with the number of days of coughing spasms, especially when no other symptom was required by the case definition. This result has also been seen for JNIH-7 in an analysis of disease verified by either culture or serology (9). For cases with



No. of Cases													
Culture	JNIH-7	15	12	6	4	4	4	0	0	3	3	0	0
Conf.	Placebo	37	35	31	26	32	30	26	22	26	27	24	21
All	JNIH-7	213	116	60	34	33	26	6	7	17	13	5	5
Cases	Placebo	151	107	73	54	57	46	35	25	46	36	30	23

* *with whoops*
** *with whoops and >=9 spasms on at least one day*

FIG. 2. Vaccine efficacy, JNIH-7: n = 1,403 (JNIH-7), 923 (placebo).

a specified duration of coughing spasms, efficacy was higher when whoops were part of the definition; it was higher still when both whoops and nine or more spasms in a day were required.

For any particular clinical case definition, efficacy was always higher when culture confirmation was required. Estimated efficacy against any illness with 21 days or more of coughing spasms was 41% for the two-component vaccine JNIH-6 and 27% for the single-component vaccine JNIH-7; the corresponding estimates for culture-confirmed disease were 81% and 75%. However, when whoops and at least nine spasms in a day were added to the case definition, efficacy was 76% for JNIH-6 and 77% for JNIH-7 against clinical illness and 87% and 92% for the two vaccines, respectively, against culture-confirmed illness.

JNIH-6 had a higher estimated efficacy than did JNIH-7 against illness with at least 14 days ($P = 0.07$) or 21 days ($P = 0.12$) of coughing spasms; its efficacy was also somewhat higher against culture-confirmed disease. However, JNIH-7 had a higher efficacy against illness with 28 days or more of coughing spasms than did JNIH-6, and its efficacy against illness with whoops was higher for all durations of coughing spasms tested. The higher efficacy for JNIH-7 was statistically significant for illness with at least 28 days of spasms and whoops, regardless of whether culture confirmation was required.

DISCUSSION

There is no single "vaccine efficacy" for JNIH-6 or JNIH-7. Efficacy estimates vary widely, depending on (i) the severity of clinical symptoms required by the case definition, and (ii) if laboratory confirmation is required. Efficacy's dependence on case definition is one of several explanations for the variation in efficacy estimates obtained in different studies (4).

The higher observed efficacy for the two-component vaccine against illness with at least 14 or 21 days of coughing spasms is consistent with previous reports (1, 9). However, the single-component vaccine JNIH-7 had higher efficacy against illness with coughing spasms of longer duration and against illness accompanied by whoops. The latter finding could be due to the higher concentration of PT in JNIH-7 (12.0 µg protein nitrogen per ml compared to 7.5 µg for JNIH-6 [1]). No data from the trial are available to further test such a conjecture.

For both vaccines and all clinical case definitions used, higher estimates of efficacy were obtained when culture confirmation was required in addition to clinical symptoms. A possible explanation for this is nonspecificity of the clinical definition, i.e., inclusion of cases which were not due to *Bordetella pertussis*. Another possible explanation is a lower probability of obtaining a positive culture for infected children who are vaccinated than for those who are unvaccinated. An earlier report suggests, however, that any such overestimation of efficacy should be relatively small for illness with more than 3 weeks of coughing spasms (9). Further analysis of clinical illness is being done, using information on exposure to *B. pertussis* as well as serological diagnostic criteria which have been shown to be nearly 100% sensitive for culture-confirmed cases (9). Preliminary results of this analysis indicate that for episodes with at least 21 days of coughing spasms, much of the discrepancy in efficacy may be due to the inclusion of false positives among the clinical cases (data not shown). Thus, efficacy against clinical illness is probably underestimated in this report.

Differences in case definitions, populations studied, and other methodological factors make it very difficult to compare the efficacy of acellular pertussis vaccines with licensed whole-cell vaccines using currently available data. Consequently, the adequacy of protection conferred by an acellular vaccine can best be assessed by directly comparing it with that offered by a licensed whole-cell product in a randomized double-blind trial. Such a trial would be costly, requiring thousands of infants and taking several years to complete.

The choice of case definition(s) would be crucial to the success of such a trial. A case definition that is not specific for pertussis would incorrectly identify illnesses which are actually due to other causes as pertussis. This misidentification could lead to a false conclusion that different vaccines are similar in efficacy or to an underestimate of efficacy when comparing vaccinated and unvaccinated children. Either type of comparison thus needs a very specific definition. Culture is specific for pertussis but is often not positive when pertussis is present; further analysis of only culture confirmed illness may lead to overestimates of efficacy, especially when milder illnesses are included (9).

A clinical definition may be appropriate for an investigation of an outbreak or of illness after household exposure when the intensity of exposure would be relatively high for most individuals (8). However, the present results suggest that for a population-based study, clinical criteria alone are not specific enough to define pertussis. Rather, the best definition of disease for such a study would combine clinical and laboratory criteria. Both culture and serology have been used in evaluating vaccine efficacy from the Swedish data (1, 9). Serological criteria for a new trial would need to be specifically developed since the criteria used with the Swedish data were developed by evaluating intra-assay variability in that laboratory setting and may not be appropriate for a different trial. The importance of laboratory methods in diagnosing pertussis suggests that, if feasible, an active surveillance system would be most desirable for a future trial, so that cultures and blood samples could be obtained early in illness or soon after expected exposure.

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New Conclusions and Lessons Learned from the Vaccine Trial in Sweden

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The efficacy of pertussis vaccines has been under scrutiny since the 1950s. Fine and Clarkson (2), in their review of the efficacy of whole-cell vaccines, stress some areas which remain controversial. They argue that "conventional, killed, whole-cell vaccines are more effective in protecting against disease than in protection against infection per se." They base their argument on several factors. First, clinical pertussis is less severe in vaccinated than unvaccinated individuals. Second, the immunological response to conventional pertussis vaccines predominantly involves immunoglobulin G (IgG) and fewer IgA antibodies; this pattern is consistent with better protection against the infection's later morbidity-associated stages than against its initial colonization stages. Finally, pertussis epidemics occur cyclically depending on the accumulated number of susceptibles in a population. If vaccines were to protect against disease much more than against infection, the frequency of cycles would not be much affected by vaccination: this lack of effect was in fact observed in both England and Sweden after the introduction of a general vaccination against whooping cough.

Fine and Clarkson emphasize the effect of case definition and case ascertainment on observed efficacy. Thus, the more clinical severity that is required for a case to be accepted as pertussis, the higher the observed efficacy will be. Also, the protective efficacy of whole-cell vaccine is higher against bacteriologically proved cases. Fine and Clarkson argue that the combined effect of these circumstances tends to overestimate efficacy; on the other hand, the inclusion of nonpertussis illness tends to reduce the observed estimate of efficacy. They conclude that the effect of different biases in reported studies is, in general, difficult to assess.

Fine and Clarkson also address the number of doses given as related to protection over time. They state that the small amount of available data suggest a progressive increase imparted by the first three doses of whole-cell vaccine, but that (to their knowledge) there are no data on the protective implications of further booster doses.

Serological correlates to protection in whole-cell vaccine recipients have been studied in the past. One study (4) suggested that a post-vaccination agglutinin titer above a certain limit conferred absolute immunity in household exposure. Recent studies have neither confirmed nor refuted this observation, which remains a basis for current recommendations by some authors to use agglutinin responses in evaluating vaccine efficacy (6).

Using salient points in the Fine and Clarkson paper as a frame of reference, this article reviews results from the randomized double-blind placebo controlled clinical trial of two acellular pertussis vaccines carried out in Sweden in 1986 to 1987 (1). The report also takes into account the subsequent nonblinded post-trial follow-up (3) and additional analyses (5) of the original data set.

Protection against disease and infection. In the efficacy trial of acellular pertussis vaccines in Sweden (1), protection against culture-confirmed pertussis including any duration of cough, was estimated to be 69% (95% confidence interval, [CI] 47 to 82%) for the two-component vaccine JNIH-6 containing toxoided pertussis toxin (PT) and filamentous hemagglutinin (FHA) and 54% (95% CI 26 to 72) for the monocomponent toxoided PT vaccine JNIH-7. Three estimates were made during 15 months of blinded follow-up from 30 days after the second trial dose. For more severe culture-confirmed cases, both vaccines were approximately 80% efficacious. According to Storsaeter, the toxoid vaccine did not protect against pertussis infection *per se*, but the protective efficacy increased with increasing severity of the clinical case definitions. The addition of FHA seemed to provide more protection against pertussis infection. These observations emphasize the different roles of the vaccine components and suggest a continuum of protection against infection from monocomponent toxoid vaccine to whole-cell vaccines.

Storsaeter showed that the efficacy of the toxoid vaccine estimated against pertussis infection was lower when using a diagnosis based on serology than when using a diagnosis based on culture. The overestimate of efficacy for culture-confirmed cases was less prominent for more typical whooping cough. As Blackwelder showed, for clinically defined cases without laboratory confirmation, the inclusion of nonpertussis illnesses seems to reduce the observed efficacy of the two acellular vaccines for typical disease. Thus, the use of clinical case definitions alone tends to underestimate and the use of culture confirmation tends to overestimate efficacy.

When using different case definitions, the different efficacies observed for the two acellular vaccines clearly emphasize the cautions noted by Fine and Clarkson in interpreting efficacy results and indicate the need for carefully delineated objectives in studies of vaccine protection. Should pertussis vaccines protect against typical or severe disease or should the vaccines also protect against infection or bacterial colonization? Fine and Clarkson seem to argue in favor of the latter. However, given the poor effect of whole-cell vaccine against colonization and infection, it is unlikely that acellular vaccines with only a few components will confer near-complete protection against infection.

Protection over time. Protection over time was studied through unblinded surveillance of the 1986 to 1987 trial cohort during the post-trial period from August 27, 1987, until March 11, 1990. All laboratory confirmed pertussis cases among study children born March through September 1985 were identified from reports made to the Swedish National Bacteriological Laboratory (courtesy of V. Romanus and Gun Sandzelius) and from spontaneous parental reporting. Study nurses interviewed parents about the course of illness in the culture-confirmed cases. Parents were asked by letter every 6 months if their child had had pertussis since August 1987. Of the 3,529 still eligible families, 3,272 (93%) responded to the latest mailing in March 1990. Nonresponders were included in the denominators of incidence rates of parentally reported illness.

The estimates of cumulative incidence (calculated by the actuarial method) in the two vaccine groups and in the unvaccinated group were used to estimate vaccine efficacy in the post-trial follow-up. During post-trial follow-up, 27 cases of culture-confirmed pertussis with cough of any duration occurred in the JNIH-6 group (*n* = 1,365), as compared to 41 cases in the JNIH-7 group (*n* = 1,370) and 71 in the placebo group (*n* = 882). Efficacy of JNIH-7 was estimated to be 65% (95% CI 49 TO 76); the efficacy of JNIH-6 was estimated to be 77% (95% CI 64 to 85). For culture-confirmed cases with more than 30 days of cough (JNIH-7, 20 cases; JNIH-6, 8 cases; and placebo, 61 cases) efficacy for JNIH-6 was 92% (95% CI 83 to 96) and for JNIH-7, 79% (95% CI 66 to 87). The data indicate that two doses of acellular pertussis vaccine provide protection for at least 3 years. The need for a booster dose is still not evident.

According to the parental questionnaires, 163/882 placebo, 46/1,365 JNIH-6, and 71/1,370 JNIH-7 recipients had whooping cough, as diagnosed by the parents, during the post-trial follow-up. Vaccine efficacy based on the parental reports was 82% (95% CI 75 to 87) for JNIH-6 and 72% (95% CI 64 to 78) for JNIH-7. Nonblinded follow-up, relying on parental reports of pertussis in conjunction with surveillance of laboratory data, thus yields similar vaccine efficacy estimates for the acellular vaccines as those observed in studies on the efficacy of whole-cell vaccines, using similar case definitions and case ascertainment techniques (2). Such historical comparisons should be regarded with caution and need to be substantiated in controlled studies.

Need for new efficacy trials. It may be concluded that (i) both acellular pertussis vaccines seem to protect against typical illness to the same extent as whole cell vaccines and (ii) protection against infection seems to be augmented by adding one or more components to a PT-based vaccine. However, these conclusions are only tentative and need to be tested.

Despite definite proof that the acellular vaccines conferred significant protection (1), no serological indicator of protection in the individual child has been demonstrated. Romani et al. showed that the two acellular vaccines did not elicit an agglutinin or antibody response to relevant antigens other than PT and FHA. Until an immunological marker of protection has been defined — whether humoral, secretory, or cellular — new acellular vaccines need to be tested in efficacy trials.

The main objective of future trials should be to estimate the protection of acellular pertussis vaccines against typical disease compared to whole-cell vaccine and placebo. A secondary objective should be to estimate the relative protection among vaccines against infection *per se*.

As discussed by Storsaeter and Blackwelder, differences in the sensitivity and specificity of various pertussis case definitions in vaccinated and unvaccinated children have important implications for upcoming trials of acellular vaccines. Given this fact, which case definition provides optimal sensitivity and specificity, according to the present understanding of clinical, serological, and bacteriological criteria?

Clinical diagnosis alone increases sensitivity, but with an accompanying loss of specificity. Thus, clinical criteria alone are not considered acceptable even for very severe cases. Requiring a positive culture or significant rise in PT antibodies increases the specificity, but is associated with a differential sensitivity: proportionally fewer cases are detected among vaccinees. This bias may be reduced by restricting the analysis to clinically typical cases. Diagnosis based on antibody rise against FHA seems to be less influenced by differential sensitivity, but does not discriminate between *Bordetella pertussis* and *B. parapertussis* infections.

Suggested case definitions. Considering the limitations of each available measure, an optimal case definition for the main objective (typical disease) would be (i) 21 days or more of spasmodic cough and (ii) either culture confirmation of *B. pertussis* or a significant antibody rise against FHA IgG or IgA or PT IgG in paired sera starting 30 days after the second or third trial dose. An optimal definition for the secondary objective (infection *per se*) would be either a positive culture, or a significant antibody rise against PT IgG, or a significant antibody rise against FHA IgG or IgA in a child with close contact with laboratory-confirmed *B. pertussis.*

In the continued evaluation of new acellular vaccines, it is best to keep in mind, as per Fine and Clarkson; advise (2); the complexity of pertussis immunity.

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DISCUSSION

DR. CHERRY: This is for Jan. On looking at your antibody evaluation of disease, it seems to me looking at agglutinins would have been a much simpler and more accurate way.

DR. STORSAETER: I think the question was that we should have used agglutination instead of these antibodies. The reason was that we used capillary sera so that very small amounts of sera were available and that microagglutination would have, be too costly in terms of serum amounts.

DR. BERNIER: Dr. Olin since we all know that 69 and 54 percent are two numbers that are emblazoned on our consciousness, some of us would like to think that there are other estimates that are perhaps better estimates to use, but there is disagreement about which of those. We certainly have seen so many more estimates of vaccine efficacy today that in an attempt to clarify things we may end up confusing them more. You are now proposing another case definition for our next trial. It seems to me it would be interesting to know if you use that same definition for the previous trial, could we not think about that as the best definition for the Swedish trial? If you apply this new proposed definition to the past trial, it would seem to me that the best estimate of efficacy, considering sensitivity, specificity and potential biases, would be somewhere around 80 percent. Do you agree with that?

DR. OLIN: I agree that we need to establish a new definition and we are suggesting the definition just stated. With that definition you arrive at approximately 80 percent, but I think it is very important to get a consensus before you start looking at the figures.

DR. GUPTA: Dr. Olin pointed out that the efficacy of whole cell vaccine taking different criteria for the severity of the disease and other criteria was similar to the acellular vaccine, but I think the efficacy of the whole cell vaccine was determined after three doses and the acellular vaccine only after two doses were used. So we could use three doses of the acellular vaccine and get better protection.

DR. OLIN: I think we should agree that we cannot make very direct historical comparisons. We have to be very careful in making a direct comparison. That is why we are arguing for a direct comparison in the prospective trial. What I tried to point out was that there are great similarities in the difficulties in interpreting data from whole cell vaccine and acellular vaccines.

With regard to two or three doses, I think the interesting observation we have is that we do not see waning immunity with time given only two doses of acellular vaccine and I think it is also interesting to note that the Japanese pediatricians have decided to go from three to two doses because of trying to keep down the number of doses given because of local reactions coming up with more doses. Although I understand the general sentiment in this country to keep three doses, there are data suggesting there needs to be discussions of whether three doses are really necessary. In upcoming trials I agree that three doses should be used and it will be interesting to look at the differences in the data from these two different types of dose schedules. So we have to wait some years to get that answer.

DR. GUPTA: Dr. Olin, you said that double blind trials will be done with acellular and whole cell vaccines. I wonder how you can keep these trials blinded because we will expect reactions after whole cell vaccine and everybody would know which have received the whole cell vaccine and the acellular vaccine.

DR. OLIN: I think you are right, there will be a problem with blinding over time which we cannot completely control. On the other hand, I do not think that this is a crucial issue, at least in the Swedish community. With regard to whether you believe you are protected or not in the direct comparisons between vaccine groups, this bias might not have so much importance. In the comparisons with the placebo and the whole cell vaccine, data have to be scrutinized very carefully.

DR. SIZARET: Which criteria would you use to select the whole cell vaccines which you will include in your new trial because you know that there may be marked differences between the existing whole cell vaccines. So the term whole-cell vaccine in itself does not really mean much.

DR. OLIN: I think this is a question which is important but prematurely raised. I think that from the Swedish point of view, we have experience with the British whole cell vaccine. That is the vaccine that is presently induced under individual licensure in Sweden and from that perspective, I think it is easier for us to argue to use that vaccine from the same manufacturer. Additionally, I think this is one of the whole cell vaccines which have the best data collected through the British studies, Elizabeth Miller's studies, etc., but this is an open question still.

DR. BERNIER: An important question which is on all of our minds has to do with the selection of the antigens for the next trial. We have four antigens that are most discussed. For the first time we are now hearing Patrick and Jan say these two vaccines are not entirely equivalent and I think you presented data to show differences in the performance of these two vaccines. At the mild end and at the severe end there seems to be differences in the performance of these vaccines. I would like to ask you, in this "animal model" what have we learned about which vaccines would be most important to include or which antigens would be most important to include in the next round of trials.

DR. OLIN: I think this has to do with what type of end points you decide on. If you use the end points suggested in the slide where the primer end point typical illness, you might argue that a single component toxoid vaccine is sufficient. On the other hand, we have clear indication that additional protection against infection and colonization will be achieved by addition of one or more antigens. So for trial purposes I think it is scientifically valid to use whole cell vaccine and monocomponent vaccine and one or more multi-component vaccines in a direct head-to-head comparison. Otherwise, we will continue to have this discussion; was it enough or not.

DR. CAMERON: I wonder if there is a retrograde step in process here. As far as I am concerned, it is almost traditional to inject adsorbed vaccines intramuscularly. I was disappointed to notice that the continued use of subcutaneous injection with the Japanese vaccine and now in the recent trial in Sweden. Considering we are talking about infants only months old and that the reactions of parents as well as infants has to be considered, the concern about the persistence of local reactions, I would like to see some recommendations coming from our clinical colleagues as to whether intramuscular or subcutaneous should be preferred.

DR. OLIN: I agree that this is an important issue. At the earlier trial, we were not prepared to go against the Swedish practice at that time. I think now we are prepared to recommend intramuscular vaccination to reduce side reactions.

DR. CHEN: I know this is a pertussis symposium but as much as we are focused on pertussis, the diphtheria and tetanus toxiods are a part of it. I think we need to keep track of the toxoid response before we jump to a two dose schedule routinely. I think we need to keep track of what the long term impact of that is. We know that in the Soviet Union right now there is a diphtheria outbreak mainly because of low vaccine coverage in their children with DTP so the impact of a two-dose schedule could be very quick without us realizing it.

Preliminary Comparison of Antibody Responses and Symptoms Following Primary Immunization with British Whole-Cell and Three Acellular DTP Vaccines

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Although acellular pertussis vaccines are generally reported to be less reactogenic than whole-cell vaccines, their efficacy relative to whole-cell vaccine has not been established. The efficacy data obtained from post-licensure studies in Japan (4) and from a phase-III trial in Sweden (1) were reviewed by the British Medical Research Council, which decided that a trial should be undertaken to compare the efficacy of one or more acellular vaccines with that of the current UK whole-cell vaccine. In preparation for the phase-III trial, a double-blind randomized phase-II study was carried out with three candidate acellular vaccines. One vaccine, developed in Britain, contained individually purified pertussis toxin (PT), filamentous hemagglutinin (FHA), and Agg 2 and 3 (AGG 2/3); the second, made in France, contained individually purified PT and FHA; the third, made in Japan, contained co-purified PT, FHA and a trace of Agg 2. The objective of the study was to compare antibody responses and symptoms after administration of these vaccines with those to British whole-cell vaccine in order to establish the acceptability of the new vaccine for inclusion in the proposed phase-III trial. All vaccines were given as adsorbed DTP preparations using a 3-, 5-, and 8 to 10-month schedule. The trial was conducted under the direction of the Medical Research Council and was coordinated by the Public Health Laboratory Service Communicable Disease Surveillance Centre.

MATERIALS AND METHODS

Vaccines. The whole-cell vaccine was an adsorbed DTP preparation from a single lot (Wellcome Research Laboratories, Beckenham, England). The composition of the three acellular DTP vaccines as reported by each manufacturer is shown in Table 1. The three acellular vaccines were independently evaluated for antigen purity, toxicity, stability, immunogenicity, and protective potency at the National Institute of Biological Standards and Control; the Institute also evaluated the lot of whole-cell vaccine for toxicity, immunogenicity, and protective potency. (The results of these tests will be reported elsewhere.) All four vaccines were dispensed in identical single-dose 0.5-ml ampules and were given by deep subcutaneous injection into the anterolateral aspect of the thigh.

Study population. The subjects were infants attending immunization clinics in the North Hertfordshire Health District between March and November 1989 for primary immunization with DTP vaccine. Infants were scheduled to receive the first dose at 3 months, the second at 5 months, and the third at 8- to 10-months. The following exclusion criteria were applied: serious chronic disease, previous laboratory-confirmed pertussis, personal history of cerebral irritation or damage in the neonatal period, development delay, convulsions, or an immediate family history of idiopathic epilepsy. Any of the following symptoms or signs occurring within 48 h of the first or second dose constituted a contraindication to a subsequent dose: temperature >39.4°C; persistent screaming or high-pitched crying lasting for 3 h; shock-like episode with cyanosis, pallor, or limpness; or generalized allergic reaction. Further DTP vaccine was also contraindicated for any infant who developed a local reaction involving most of the anterolateral aspect of the thigh. Details of children with severe local reactions or

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TABLE 1. Acellular vaccines in U.K. phase 2 trial

Manufacturer	Antigen content (ratio)	Total pertussis protein per dose	Toxoiding agent	DT antigens
PHLS/CAMR	PT/FHA Aggs 2, 3 (1:1:1)	30 µg	Formaldehyde	Wellcome
Merieux	PT/FHA (1:1)	50 µg	Glutaraldehyde	Merieux
Takeda (Lederle)	PT/FHA/Agg 2 (9:90:1)	47.5 µg	Formaldehyde	Lederle

systemic symptoms were referred to an independent data monitoring committee which held the vaccine code for confidential review and decision as to whether the study should be allowed to continue. The trial was approved by the North Hertfordshire District Ethical Committee, and written informed consent was obtained from parents.

Blinding and randomization. Owing to delay in the availability of two of the acellular vaccines, the trial was conducted in two stages. In the first stage, whole-cell vaccine was compared with Public Health Laboratory Service, Centre for Applied Microbiology and Research (PHLS/CAMR) acellular vaccine; in the second stage, whole-cell vaccine was compared with the Merieux and Lederle acellular vaccines. In each stage, vaccines were randomly allocated to sequential study numbers in equal proportions, and infants were allocated to study numbers in order of attendance at the clinics. Block randomization of vaccines to study numbers was performed by a computer program. The vaccine code was not disclosed to parents or field, laboratory, or coordinating staff until after the analysis was completed.

Evaluation of reactogenicity. Infants were followed up to identify systemic symptoms and local reactions by study nurses who visited homes at 24 h and at 7 days after vaccination. The nurses measured rectal temperatures and local reactions and completed a standard symptom questionnaire. Parents monitored infants' axillary temperatures and recorded the size of any local reaction at specified times during the 7 days after vaccination.

Measurement of antibody responses. Serum immunoglobulin G (IgG) antibody responses to PT, FHA, and Agg 2/3 in capillary blood samples taken before the first dose and 6 to 8 weeks after the third dose were measured at PHLS/CAMR by enzyme-linked immunosorbent assay (ELISA) as previously described (6), but with the following modifications.

(i) ***Reference serum.*** High-titer sera from five adults, including two recent whooping cough cases, were pooled and heat-treated. The serum was ampuled and freeze dried at the National Institute of Biological Standards and Control and designated "Anti-pertussis 89/530" (Ref 89/530). Ref 89/530 was assigned titers for IgG antibodies to each of the three test antigens to allow direct comparison with results of previous studies (6).

(ii) ***Antigens.*** Antigens were made and tested by the same physiochemical techniques used for the CAMR antigens but, instead of being treated with formaldehyde, were freeze dried and stored at 4°C. The freeze-dried antigens were reconstituted in 50% (vol/vol) glycerol in water and stored at -20°C between assays. FHA was coated on assay plates at 1 µg/ml; PT and Agg 2/3 were coated at 2 µg/ml.

While the test antigens in the ELISAs were purified at CAMR by the same procedures and from the same bacterial strain used to produce CAMR vaccine antigens, three preliminary studies were carried out to investigate whether these antigens were sufficiently representative that they would not adversely affect the measurement of antibody responses to the Merieux and Lederle vaccines.

A set of 45-murine sera raised against the Merieux vaccine was tested for antibodies to PT and FHA both at CAMR using the CAMR antigens and by Merieux using an ELISA in which it prepared the coating antigens (3). There was a linear relationship between log titers obtained by CAMR and Merieux for both FHA and PT; there were no outliers. Of the 45 sera, 12 had undetectable titers to FHA and PT in both the CAMR and Merieux ELISAs. The correlation coefficients for the samples with positive titers were 0.85 (95% confidence interval [CI] 0.76 to 0.94) for FHA, and 0.83 (95% CI 0.72 to 0.94) for PT.

A set of 49 sera from children receiving the Lederle acellular vaccine for primary immunization was tested for antibodies to PT and FHA both at CAMR and by Lederle using their in-house ELISA. There was a linear relationship between log titers obtained by CAMR and Lederle for both FHA and PT with one significant outlier for the PT titers. The correlation coefficients were 0.76 (95% CI 0.64 to 0.88) for FHA, and 0.72 (95% CI 0.59 to 0.85) for PT.

The CAMR working standard and a set of 23 sera from whole-cell vaccines, unvaccinated children, and children with whooping cough were tested for IgG antibodies to PT and FHA by ELISA at CAMR and Lederle and for antibodies to PT at Merieux. ELISA titers from all three laboratories were corrected to the same unitage using the CAMR working standard. Linear relationships were obtained between log titers for antibodies to PT assayed by CAMR and Merieux, antibodies to PT assayed by CAMR and Lederle, and antibodies to FHA assayed by CAMR and Lederle with correlation coefficients of 0.83, 0.80, and 0.86, respectively. The slope of the regression line of CAMR on Merieux titers to PT was 1.0, of CAMR on Lederle titers to PT was 1.0, and of CAMR on Lederle titers to FHA was 1.05. All three regression lines had intercepts not significantly different from zero.

TABLE 2. Number of infants recruited into the trial who completed the course, and the number who failed to complete because of a contraindication

Trial stage	Recruited	Completed (%)	Contraindication to further dose
Stage 1			
Whole cell	94	88 (94)	1
CAMR acellular	94	91 (97)	1
Stage 2			
Whole cell	85	82 (96)	2
Merieux acellular	74	69 (93)	4
Lederle acellular	85	78 (92)	3
TOTAL	432	408 (94)	11

The above results indicated that use of CAMR antigens as test antigens was unlikely to bias the comparison among acellular vaccines.

(iii) *Assay layout.* Pre- and post-vaccination sera from each individual were assigned in random order to the same assay plates. A single eight-well, threefold dilution series was made for each serum and 50-μl aliquots from this series transferred to the three washed antigen-coated plates. Diluent was added (50 μl/well) to yield an overall 108-fold dilution of serum in well 1 of each titration. Ref 89/530 was titrated twice (from 720-fold dilution in well 1) on each plate as a working standard.

Each serum sample from the phase-II trial was coded before being assayed at CAMR. Antibody results of the coded sera were sent to the Communicable Disease Surveillance Centre for collation and analysis.

Statistical considerations. The number of vaccinees per group (at least 80) was chosen to ensure a high probability that material differences in reactogenicity and immunogenicity between the whole-cell and acellular vaccine groups would be detected. Log antibody titers were approximately normally distributed. Comparisons of baseline variables, frequency of reactions, and log titers were performed by means of a two-tailed *t*-test or chi-square test. ELISA results from different laboratories were compared using regression techniques and correlation coefficients.

TABLE 3. Comparison of systemic symptoms and local reactions following Wellcome whole cell (A), CAMR acellular (B), Merieux acellular (C) and Lederle acellular (D) DTP vaccines (all 3 doses combined)

Adverse event within 24 hours	A Wellcome (%)	B CAMR (%)	C Merieux (%)	D Lederle (%)	*P* Value A:B	A:C	A:D
Rectal temperature ≥38°C	61/503 (12)	8/263 (3)	7/198 (4)	2/233 (1)	<0.001	<0.001	<0.001
≥3 symptoms[a]	120/523 (23)	25/278 (9)	35/216 (16)	21/244 (9)	<0.001	0.041	<0.001
Local erythema	227/501 (45)	140/262 (53)	86/205 (42)	88/236 (37)	<0.033	0.41	0.040
Local swelling	258/497 (52)	133/262 (51)	65/203 (32)	61/233 (26)	0.76	<0.001	<0.001

[a] Feeding less, disturbed night, irritable, sleepy, crying more than usual, vomiting.

TABLE 4. Proportion of infants with rectal temperature ≥38° at 24 hours according to vaccine and dose.

Dose	Whole cell vaccine (%)	All acellular vaccines (%)	P value Whole cell:Acellular
1st	6/170 (4)	2/243 (1)	0.05
2nd	14/168 (8)	10/227 (4)	0.11
3rd	41/165 (25)	5/224 (2)	<0.001
TOTAL	61/503 (12)	17/694 (2)	<0.001

RESULTS

Of the total 432 infants recruited into the study, 408 (94%) received all three doses. The numbers of infants that were recruited and that completed the course in each vaccine group are shown in Table 2. Fewer infants received Merieux vaccine since a substantial number of ampules was lost due to problems with clinic refrigerators. It was therefore necessary to re-randomize vaccines to study numbers, with a lower proportion allocated to the Merieux vaccine towards the end of the second stage of the trial. Of the 24 infants who failed to complete the course, 11 had symptoms within 48 hours of a first or second dose which contraindicated further vaccine; these infants were not clustered in one vaccine group (Table 2).

There was no significant difference between the two whole-cell groups and the three acellular groups in terms of sex ratio, mean birthweight, family size, or age at vaccination. In the following analyses, data from the two stages of the trial have therefore been combined.

Adverse events. The proportion of infants with rectal temperatures ≥ 38°C at the 24 h follow-up was lower after each of the acellular vaccines than after whole-cell vaccine (Table 3). With the latter, pyrogenicity increased markedly with successive doses (Table 4). Axillary temperatures taken by parents showed that temperatures after whole-cell vaccine were highest on the second day

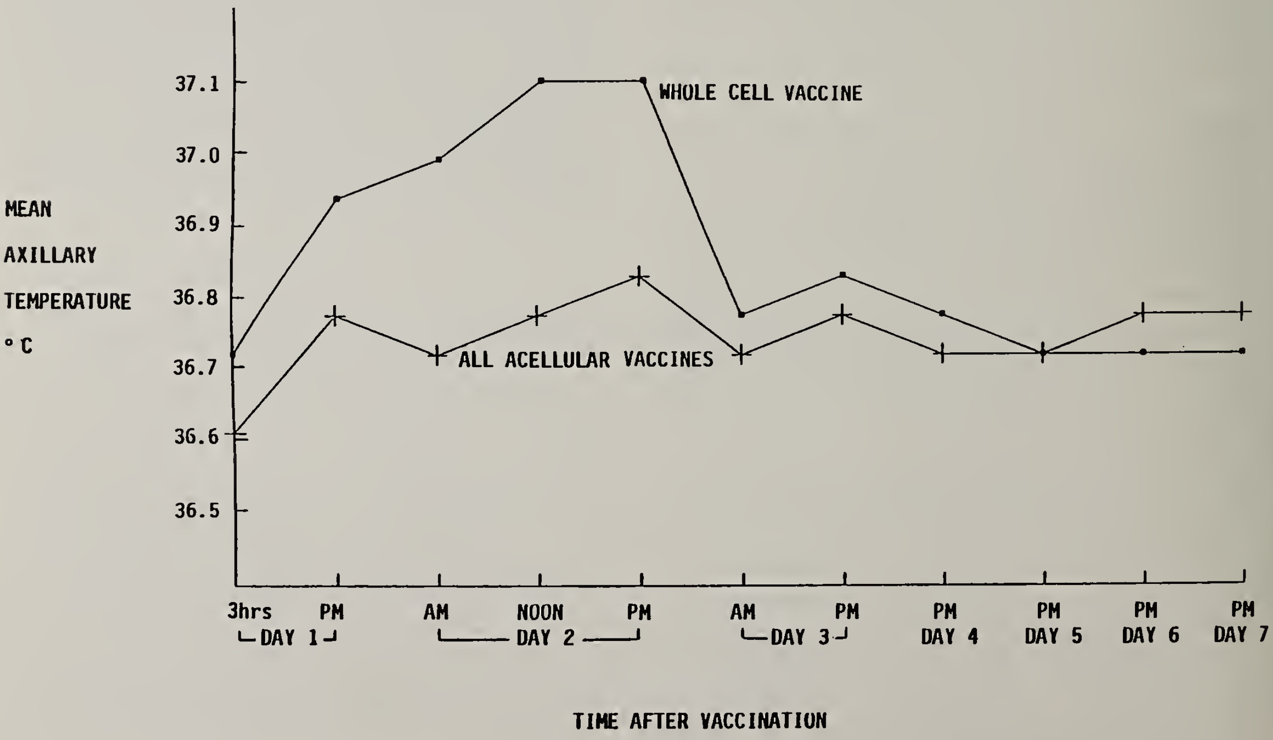

FIG. 1. Infants' mean axillary temperatures taken by parents during the first week after vaccination (third dose).

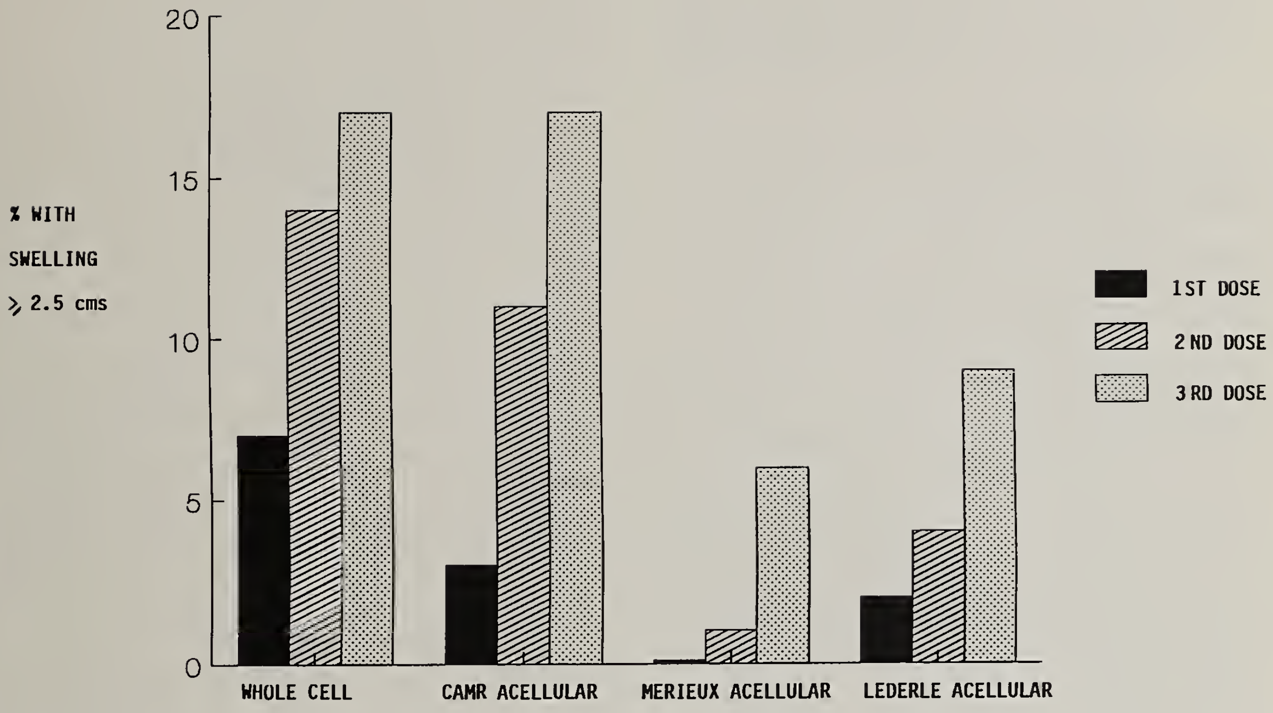

FIG. 2. Swelling ≥ 2.5 cms at injection site according to vaccine and dose: parental reports at 24 hours.

after vaccination (Fig. 1). Other systemic symptoms, such as excessive crying, irritability, vomiting, anorexia, and sleep disturbance, were also reported significantly less frequently at the 24-h follow-up after the three acellular vaccines than after the whole-cell vaccine (Table 3).

The proportion of infants with local erythema and swelling was lower after the Merieux and Lederle acellular vaccines than after the whole-cell or CAMR acellular vaccines (Table 3). With all four vaccines, local reactions increased with dose number (Fig. 2). Parental reports confirmed that both frequency and size of local erythema and swelling were maximal at about 24 h after vaccination.

A total of 21 infants was admitted to the hospital with respiratory, gastrointestinal, or urinary infections during the period between the first dose and 6 weeks after the third dose. Of these, 4 had received whole-cell, 5, CAMR acellular; 1, Merieux acellular; and 11, Lederle acellular vaccine. No case of invasive bacterial infection was reported in the trial infants.

Antibody responses. Of the 408 children who completed the course with trial vaccine, 384 (94%) had a serum sample taken after the third dose and tested for antibodies to FHA, PT, and Agg 2/3. The mean interval between vaccination and sampling was 55 days. Fig. 3 shows the mean $\log_{10}$ post-third antibody titers and its 95% CI for each antigen in each vaccine group. Antibody responses to PT and Agg 2/3 after the CAMR vaccine were significantly higher than after the whole-cell vaccine; responses to FHA were similar in the two groups. Both the Merieux and Lederle vaccines gave significantly higher antibody responses to FHA, and significantly lower responses to Agg 2/3, than did whole-cell vaccine. The Agg 2/3 antibody levels after the third dose of the Merieux and Lederle vaccines were significantly higher than the levels in these groups before the first dose. The PT antibody response to the Lederle vaccine was similar to that after whole-cell vaccine; to the Merieux vaccine, it was similar to that after thCAMR CAMR vaccine.

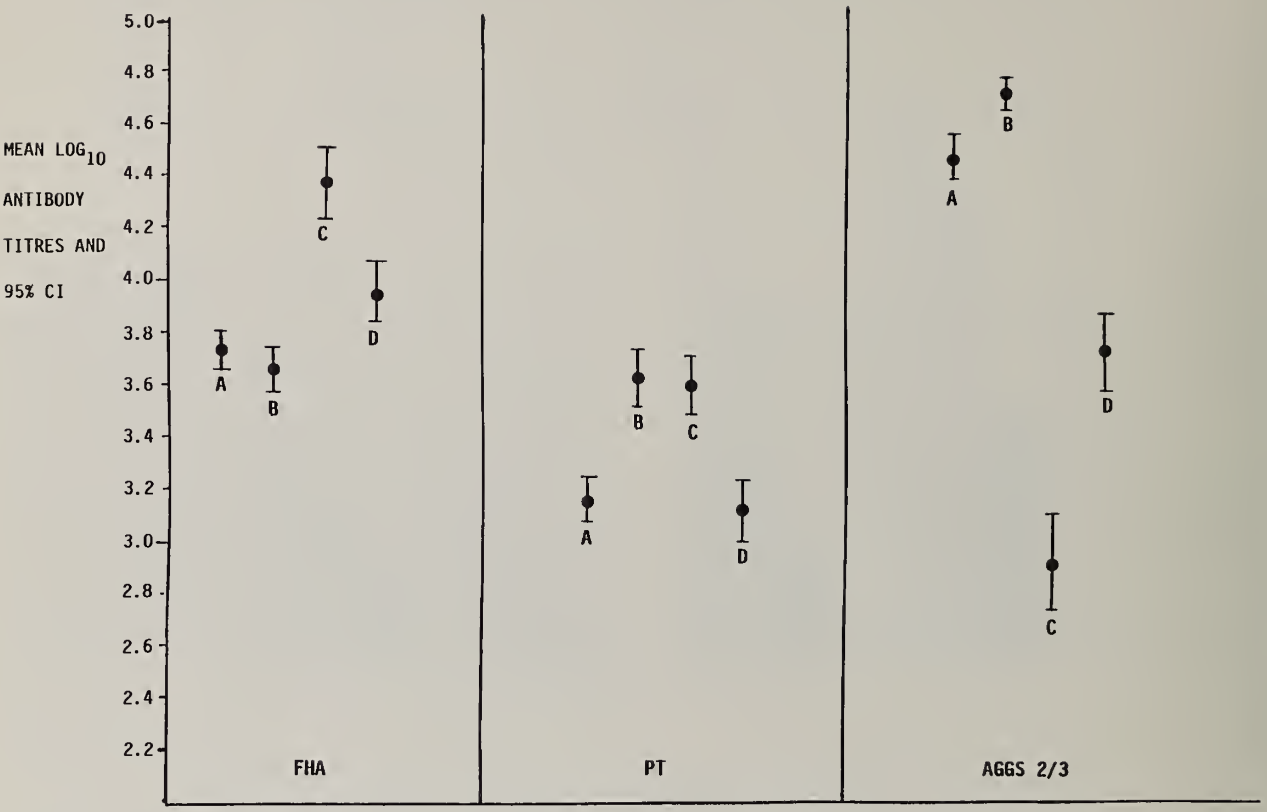

FIG. 3. Mean $\log_{10}$ IgG antibody titers and 95% CI after the third dose of Wellcome whole-cell (A), and CAMR (B), Merieux (C) and Lederle (D) acellular vaccines.

DISCUSSION

The results confirm the low reactogenicity of acellular preparations relative to UK adsorbed whole-cell DTP vaccine. With the exception of local reactions, there were no marked differences in reactogenicity rates among the three acellular vaccines. The proportion of acellular vaccinees with fever (temperature ≥ 38°C) at 24 h was similar to that found in a Swedish phase-II trial in which a Japanese PT/FHA vaccine was given to 6-month-old infants (2). The adsorbed whole-cell DTP vaccine used in the present trial produced substantially fewer fevers than the plain whole-cell vaccine used in the Swedish trial.

The incidence and size of local reactions after the CAMR acellular vaccine were similar to those after the whole-cell vaccine; both these vaccines contained Wellcome DT components. This finding is consistent with those of an earlier study which showed that local reactions after Wellcome DTP and DT vaccine were similar (5).

Significant differences in antibody response to either PT, FHA, or Agg 2/3 were found among the acellular vaccines and between each acellular vaccine and the whole-cell vaccine. With the exception of Agg 2/3, there was no clear relationship across acellular vaccines between antigen amount and antibody response. For example, 10 µg of CAMR PT produced similar antibody levels as 25 µg of Merieux PT; 25 µg of Merieux FHA produced significantly higher antibody levels than 40+ µ of Lederle FHA. The CAMR vaccine was the only acellular vaccine that produced antibody levels to all three antigens which were at least as high as those after whole-cell vaccine. The Merieux vaccine was not reported to contain agglutinogens, but a small antibody response was measured by the Agg 2/3 ELISA. An agglutinin response to the Merieux vaccine has also been demonstrated using a microagglutination assay (3).

This trial has established that each of the three acellular vaccines is significantly less reactogenic and at least as immunogenic, with respect to PT and FHA, as UK whole-cell vaccine when given at 3, 5, and 8 to 10 months of age. Each acellular vaccine is therefore a potential candidate for the proposed phase-III trial. In May 1990, however, the UK primary immunization schedule for DTP changed to a 2-, 3-, and 4-month schedule. Further phase-II trials to compare the immunogenicity and reactogenicity of candidate acellular vaccines and whole-cell vaccine given according to the accelerated schedule are therefore required so as to decide which acellular vaccine(s) should be included in the phase-III trial.

ACKNOWLEDGMENTS

This study was funded by the British Medical Research Council.

The authors gratefully acknowledge the help of C. Andrews and A. Thompson, who performed the ELISAs; M. Judd, Director of Public Health, North Hertfordshire Health Authority; and the study nurses, M. Heath, M. Hobbs, L. Joslin, T. Till, and M. Wheal. We also thank P. Farrington for his help with the statistical analysis and Wellcome for the gift of whole-cell DTP vaccine.

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DISCUSSION

DR. MEADE: What kind of distribution and absolute amounts did you see in the pre-immunization levels compared to, for example, the whole cell vaccine or the PT and FHA?

DR. MILLER: The distributions?

DR. MEADE: Yes, the distribution and the geometric mean for the pre-immunization group.

DR. MILLER: I do not have those data here. I do not know whether Dr. Ashworth can help us.

DR. MEADE: The responses you mentioned were as titers and I was trying to tie them to some other numbers that we had. One way would be to look at the pre-immunization levels to know where they fell in relationship to the post-immunization levels for the the whole cell vaccine, for example.

DR. ASHWORTH: I do not have those data with me but we do hope to standardize the results from this trial so they will be directly comparable with those from elsewhere in terms of use because you know there are several standard sera around the world and no agreement. The Swedes have their own, we have a reference we use and you have your own. Until those are brought into line, we thought that we would stick to reporting titers, but unfortunately I do not have the pre-immunization titers with me.

DR. EDWARDS: Could you please define your local swelling and erythema in terms of extent or size so that we could make comparisons? Were there any severe reactions in any of the vaccine recipients such as seizures or other neurologic events?

DR. MILLER: The answer to the first question is in the slide showing you the differences in the increase in the size of swelling at the injection site, according to vaccine dose. We were referring there to swelling of at least 2.5 centimeters at the injection site but we also had parental reports of any swelling. The second question was in relation to seizures or other serious neurologic reactions. The answer is none was reported but on the other hand, of course, we were dealing with very small numbers and one could expect on chance to have encountered any events of that sort.

DR. CHERRY: I would like to follow up on that. I think you showed 11 children who were thought to have contraindications to further immunization and I did not see the breakdown. What were those illnesses?

DR. MILLER: I have not got the breakdown on that here, Dr. Cherry, I am sorry.

DR. MINK: Were you able to show any correlation with the ELISA fimbrial titers to agglutinin titers done by microagglutination?

DR. MILLER: May I refer that to Dr. Ashworth?

DR. ASHWORTH: We have performed such studies on other groups of sera but not on these. There was insufficient volume from the samples we were given to perform microagglutinations, so we did not attempt to do it.

DR. MINK: One recommendation for performing microagglutination is to start at 25 microliters at a one to four dilution. Anything below that probably is not biologically significant and maybe doing the agglutinin titers will provide additional information.

DR. ASHWORTH: Just to put it into context, the IgG antibody assays are done on five microliters of serum and though I know what is asked for in the present US phase two trial, it is quite a large volume of blood. We were not able to achieve anything like that volume in the phase two UK trial.

DR. LAMB: Have you looked at IgE response against any of these antigens and if so, is there a correlation with reactogenicity?

DR. MILLER: The simple answer is no.

DR. Y. SATO: Is there any agglutinogen in Merieux' vaccine?

DR. MILLER: Not that I am aware of, is that correct, Ed? Not supposed to be, Dr. Sato.

DR. ASHWORTH: There is a slight antibody response within the Merieux vaccine group to the fimbrial antigens. We cannot explain it, this has been reported in studies on that vaccine in the United States and the reasons have been explored but there is no explanation forthcoming.

DR. MILLER: It is a fact that the antibody levels for agglutinogens two and three are for the third dose of both, Merieux and Lederle, were significantly higher than the antibody levels in those groups before the first dose but we do not have any explanation for that.

DR. MEADE: Would you like to respond to that, Dr. Quentin-Millett?

DR. QUENTIN-MILLETT: Yes, we have no agglutinogens in our FHA preparation or PT. This has been tested by monoclonal antibodies in other laboratories than ours. That is for sure. The other question that one can ask is, are you sure there is not any FHA in the agglutinogen you are using for the ELISA test?

DR. POOLMAN: What is the current situation in England with the acceptance rate of the whole cell vaccine and what does that mean for possible phase three studies?

DR. MILLER: The update rate for whole cell vaccine has been increasing steadily. Last year it was around 70 percent. This year it looks like hitting 80 percent.

DR. GALAZKA: I am not sure whether I follow the data on antibody response but it seems to me that the response was in general very good because it was not less than log three and in some routines probably even nearer to log four. I would like to come back to the question raised by Dr. Gupta to Dr. Olin about the number of doses given. Dr. Olin mentioned that there are some data showing some value of two doses but from the first presentation we have seen an extremely steep decrease of antibody between vaccination and the onset in at least six cases. I am not so sure that putting these data together with all evidence which we got do not speak for at least three doses. Then we can compare with existing knowledge of the efficacy of three doses of whole cell vaccine.

DR. GRANSTRÖM: I think we have noted in phase two studies the decrease after immunization, but in the studies made by Dr. Margareta Blennow the decrease at 17 months later were actually faster for the children who received three doses and had a higher titer. So by 17 months, irrespective of number of doses, everybody had a low antibody level. So there was no difference between two and three doses.

DR. MILLER: Can I make just one comment on that, Mr. Chairman? Simply to say that I think that these points are all very valid but of course we have also got to consider the practicalities in relation to the fact we are immunizing with DTP, not just with P and we also are in most immunization schedules are using polio as well. So that we have got to watch that we do not complicate things too much from that point of view as well.

Outstanding Issues in the Clinical Evaluation of New Acellular Pertussis Vaccines

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Approximately two dozen new acellular pertussis vaccines have been developed since the late 1970s. Six of these have actually been licensed in Japan since 1981, and over 40 million doses have now been administered to children in that country. A dozen other vaccines have been developed in Europe and North America and tested in children on these continents. Much valuable information on the safety and efficacy of these vaccines has been accumulated from these experiences.

Regarding safety, the rates of local and systemic reactions are lower than those associated with whole-cell vaccines. For more serious but very rare adverse events, comparative studies are not feasible; it is assumed that acellular vaccines will be associated with fewer of the serious events that have been associated with whole-cell vaccine. For adverse events that might occur once in every few thousand vaccinations, observations on a larger number of infants than has been studied to date are feasible and would be desirable.

Regarding efficacy, the available data from Japan provide evidence for the efficacy of acellular pertussis vaccines in children 2 years and older and suggest that the efficacy achieved is equivalent to that obtainable with current whole-cell vaccines. The efficacy of Japanese acellular vaccines has been confirmed in Sweden in infants, and data from the post-trial follow-up period have given estimates of efficacy similar to those in earlier whole-cell vaccine trials using similar methods of case ascertainment. However, without a serologic correlate of protection to compare the vaccines in young infants directly, a more definitive conclusion about the relative efficacy of acellular versus whole-cell vaccines can only come from a head-to-head comparison in another clinical efficacy trial.

Additional trials of acellular vaccines are therefore desirable to obtain greater certainty about the relative efficacy of acellular vaccines: secondarily, these trials will also gather more observations on safety. Such comparative data are important not only for licensure but also for guiding vaccine advisory groups in making their recommendations as to which pertussis vaccines are preferred for routine use in infants. At present, different governments, manufacturers, and vaccine developers share these views and are interested in conducting additional efficacy trials. For example, an interagency group of the Public Health Service led by the National Institutes of Health is now preparing for a new United States Government-sponsored trial. This report identifies major issues, most of which are common to all sponsors facing clinical trials, confronting United States organizers and suggests options for meeting these challenges. If successfully launched, the trial is expected to run for approximately 3 years, to cost several million dollars, and to provide data for possible licensure by the middle of the 1990s at the earliest.

Where should the trial be carried out? There are numerous qualifying criteria that must be considered in selecting a site. Of these criteria, the incidence rate of pertussis is the most crucial because it influences both the size and duration of the trial.

As in many other countries with high pertussis vaccination levels, the incidence of pertussis is relatively low in the United States. Therefore, a 2- to 3-year trial with study groups of a logistically manageable size is not considered feasible. A collaborative trial conducted in a foreign country is the only potentially viable option.

Another important criterion in site selection is a capacity for making a bacteriologic diagnosis of pertussis: this capacity is not always found together with a high incidence rate. Consequently, the number of qualifying countries where a trial can be carried out successfully is thought to be very small. This small number of potentially suitable populations creates competition among parties interested in conducting trials.

No site is ideal from all points of view, and any site selected is likely to involve compromises away from the ideal design. A few countries have been identified as potential trial sites, and detailed protocols have been written for some of these countries. Also, although a manufacturer-sponsored trial actually began in Senegal in the summer of 1990, it was only after a long preparatory period and pilot study. Start-up has been delayed everywhere a trial is planned because of numerous secondary, but still significant, requirements that must be fulfilled for a trial to satisfy the needs of its sponsors and host governments. For example, countries that presently use whole-cell vaccines are interested in comparing the efficacy of these against new acellular vaccines. However, some countries with high incidence do not routinely use whole-cell vaccines and may be unable or unwilling to conduct such a comparative trial. If the studies which are ultimately conducted are carried out only in the narrow interest of the immediate sponsors or host countries, these studies may become missed opportunities to answer important questions, and it may be difficult or impossible to find suitable replacement populations.

At present, planning for the new United States-sponsored trial is focusing on four potential sites. Final selection will be made in 1991.

What antigens should be included in the vaccines tested? Over a dozen unlicensed candidate vaccines now exist. These vaccines contain from one to four antigens in different combinations. There is no international consensus as to what the antigenic composition of the ideal vaccine in the next round of trials should be. Resolution of this question is one of the most pressing items facing the United States-sponsored trial.

Several factors affect decisions as to antigenic composition and may force compromises away from the ideal vaccine. For example, trials may be possible in some sites only if certain vaccines from particular manufacturers or developers are used. This factor affects not only acellular vaccines but also the choice of whole-cell vaccine, since not all whole-cell products may be considered of equal efficacy.

Evidence from the trial in Sweden suggests that (i) pertussis toxoid alone protects solidly against classical pertussis, (ii) filamentous hemagglutinin increases protection against milder disease, and (iii) an increased amount of pertussis toxoid may increase protection against pertussis with more long lasting cough. From these results, it could be argued that the vaccine of choice should, at a minimum, include pertussis toxoid similar to that contained in JNIH-7 and filamentous hemagglutinin similar to that contained in JNIH-6.

Obviously, the choice of a vaccine or vaccines for a future trial will depend in part on the number of study groups included. Ideally, a future trial would include at least four acellular vaccine groups, one of each prototype acellular vaccine starting with a monovalent pertussis toxoid and with three other bivalent, trivalent, and quadravalent vaccines, each adding one new additional component. In reality, no single site may be able to include this many groups and multinational as well as multicenter collaborative trials may be the only way to test each prototype acellular vaccine effectively.

Other factors that could affect the choice of vaccines are the anticipated availability of different products after a trial, the existence of other trials with the same antigens, and the willingness of sponsors with different interests to share the cost of a trial.

How should a future trial be designed? Although a randomized trial is rather simple conceptually, the number of possible study designs that can be introduced is large. Ideally, information on both the absolute and relative efficacy of acellular pertussis vaccines would be sought. This would necessitate the inclusion of both a whole-cell group and a placebo comparison group. Obviously, the number of countries where a placebo control would be ethical to implement is very limited. If only one control is possible, preference should be given to a whole-cell comparison group: determining relative efficacy deserves a higher priority than determining absolute efficacy for yet another acellular vaccine. However, countries with a high incidence of pertussis usually have low coverage with pertussis vaccine, and public acceptance of whole-cell vaccine may not be adequate to conduct a trial successfully.

For a study that seeks to compare vaccines, an additional problem is the very large sample sizes required in each group to determine if one vaccine is equal to or no less effective than another. Each study group may have to be 5 to 10 times the size that was included in the Swedish trial. Such a trial is not possible in all countries; wherever it is carried out, it will require considerable logistic support to be successful.

What should be the case definition for pertussis in a future trial? One of the key lessons relearned from the trial in Sweden is the importance of case definition. Estimates of efficacy vary according to the case definition analyzed, and not all definitions provide unbiased, sensitive, and specific measures of disease incidence. Furthermore, the case definition embodies the public health community's concept of what type of pertussis illness is most worth trying to protect against with vaccination. Some experts argue that the vaccine's ability to protect against both infection and disease should be measured. Although both may be important, protection against typical or classical disease is a higher priority. Some vaccines which protect better against infection may not protect as well against more severe disease and vice versa. There should be an advance consensus as to what is the most important outcome that vaccination seeks to protect against; case definition should be determined based on this consensus.

What should be the vaccination schedule used in a future trial? Assuming no serologic or other correlate of immunity is detected in a future trial, the efficacy data will be conclusive only for the age and vaccine groups studied. Since many countries do not administer all doses of pertussis vaccine by 6 months of age and may not be willing to alter their schedules to fit other interests, the United States and other countries may not obtain data on the exact age group they wish to vaccinate. Similarly, policies governing the use of antibiotics as prophylaxis in exposed young infants could restrict the age group available for study in some countries.

Another consideration in planning the schedule for future trials is the number of doses to be administered. Without a serologic or other correlate of immunity, it will be difficult to determine the optimal number of doses in advance of the trial.

Summary. Because of the large number of complex issues surrounding site selection, vaccine selection, study design, case definition, and vaccination schedule, it is uncertain if any of the several trials being planned can be successfully carried out. Furthermore, the limited number of sites and the multimillion dollar cost of any single trial make it unlikely that very many trials can be completed.

Greater coordination and collaboration among all interested parties could bring more resources to bear in problem solution, thereby helping to enhance the prospects for success. The potential benefits from increased cooperation are impressive and well worth making the effort in overcoming the many private interests of individual countries and manufacturers.

DISCUSSION

DR. BERNIER: I know there are many non-molecular biologists out there who have a lot of things on their minds who have not had a chance to speak so I cannot imagine there are no questions.

DR. MILLER: Given that there are so many constraints about undertaking the designs of the ideal trial, the constraints on the different schedules and different expectations and so on as well as the technical issues involved which you have outlined so comprehensively, speaking as a public health scientist, what do you regard as the minimum requirements that might be expected before we can safely and reasonably introduce acellular vaccine into routine immunization? I ask that deliberately as a generalization as a public health physician because I realize that the answer to that may have to be different in different countries so I would like it, if you can give us a general indication of what you personally regard as being the minimum requirements.

DR. BERNIER: There are other people here from other Public Health Service agencies that you may want to address that question to. I think I know what you are asking but let me clarify. Are you saying what data would we consider minimally satisfactory coming from the next trial?

DR. MILLER: Or group of trials, rafts of different kinds of evidence from different situations. What do you want to get out of this to enable you to be satisfied that it is reasonable and safe to proceed.

DR. BERNIER: There are other people here from FDA and NIH who also would have opinions but I would say that the critical question in front of us now is how do how do these acellular vaccines compare to whole cell vaccines. So I think the absolutely critical question is a two arm trial between one acellular vaccine and one whole cell. If I wanted to be perfectly conservative, I would pick either JNIH-6 or JNIH-7 which I know work and I would compare them to whole cell vaccines and that to me would be the minimum, the most conservative experiment. Now, what result would I like to see? I would like to see, as far as efficacy that the acellular vaccine is equal to or greater than a whole cell vaccine. Anything less than 10 percent or less, we would call it equivalent. So if whole cell vaccine is 90 percent effective, and acellular is 85 or 84 or 83 or 81, we would say it is equal. The sample size would not allow us to detect a smaller difference than 10 percent on a practical basis.

As far as safety is concerned, it is not possible to do studies to look at very rare neurologic conditions. As I tried to indicate on the slide, for these rare events we are going to have to make assumptions. Clearly if the size of the trial is very large, perhaps as much as if there were two groups of 20,000 or more; that is not a small sample and we will get some additional safety data. But I think for safety we are going to have to make an assumption that without data, if acellular vaccine is safer for the local and systemic reactions, then we are going to assume that it is safer for the more rare reactions.

DR. CAMERON: I have been making whole cell vaccines fairly successfully for 26 years and I am tempted to ask what is the problem? Without being too cynical about that, what you have really outlined is a horrendously expensive program and since most of my work nowadays is concerned with people in developing countries, there is simply no way these people will ever be able to afford the kind of vaccine we are talking about.

DR. BERNIER: I guess you are making two comments. The first, what is wrong with whole cell vaccine. I think it is true that there are strong differences of opinion about the safety of whole cell vaccine. I do not think I need to talk to this group about that. The second point has to do with the developing world. Perhaps Dr. Galazka some of the manufacturers would like to comment on that because it is not clear to me in any discussions that I have heard that we anticipate large price differentials between whole cell and acellular vaccine. Maybe that is naive on my part and I do not have any good grasp of what it takes to make an acellular vaccine and perhaps manufacturers can tell us. I am not sure what an accelular vaccine will cost.

DR. CAMERON: The fact of the matter at the moment is that a whole cell vaccine in North America for reasons best known to manufacturers I think is between $12 and $20 a dose and in developing countries it is cents.

DR. LONG: We have to remember that the efficacy data that we have on acellular vaccine is very short term. We hope and we know that the Swedish are continuing to look at that efficacy more than one or two years down the line and we know from our studies on looking at antibody responses following whole cell vaccine many, many, many years later, sometimes in residential facilities where there has not been pertussis occurring in those interim years, that that antibody response is very long lived following whole cell vaccine and we do know that following acellular vaccines, some of the antibody titers especially against pertussis toxin go down very quickly. In the very long term studies following whole cell vaccine, reliably those adults have antibody against agglutinogens and antibodies of IgG against FHA. What they do not usually have is much antibody left against PT of IgG and yet we think they are usually clinically protected. So you may make the decision on short term efficacy. We will not know the long term efficacy for many years.

DR. BERNIER: I think that is true but I think that is a limitation that we accept in any clinical trial; that we can only know the efficacy for as long as we have measured it. I think that is an extremely unique thing about the Swedish trial, that the trial has continued after the trial has ended. We must exploit this to the maximum extent possible and I think the Swedes have done a really remarkable job to continue to collect data the way they have and to now know that there is no waning immunity after four years with two doses I think is a very,very important piece of data. We are not often in a position of having the luxury to know that. It is one bright spot in the otherwise sometimes discouraging picture about the development of pertussis vaccines. We are all impatient that it seems to be taking so long but at least this is one thing we may have more than what we had with other vaccines.

Detection of *Bordetella pertussis* in Clinical Specimens

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The detection of the causative agents of whooping cough, *Bordetella pertussis* or *Bordetella parapertussis,* is the hallmark of every case definition of pertussis and is of paramount importance for the diagnosis of the disease. Most agree that healthy carriers do not commonly occur (8, 22, 25), indicating that the isolation of the bacteria is always relevant to disease. Thus, techniques for detecting *Bordetellae* or their structural components should be made as sensitive as possible while remaining feasible for the practicing pediatrician.

Patient samples. Nasopharyngeal (NP) swabs yield higher isolation rates of *B. pertussis* than the cough plates which were used in the past (24). Thus, NP swabs are the most common method for specimen collection. Duplicate swabs from both nostrils are recommended to increase the sensitivity of detection (31). Calcium alginate has been shown to be superior to other swab materials, such as dacron, which in turn was found to be superior to cotton wool (20). Throat swabs (26) or anterior nasal swabs are not useful, as *Bordetellae* have a specific tropism for ciliated respiratory epithelium, which is not found in the anterior nose and the pharynx. NP secretions aspirated by AUGERs suction or NP washings represent another suitable material to detect *Bordetellae;* these methods can be adapted for use in a pediatric office, where most pertussis cases are seen (10, 11). Although no comparative study has been made of the different sensitivities of NP swabs and NP aspirates for culture of *Bordetellae,* NP aspirates have demonstrated a higher sensitivity for other microorganisms such as RS-virus (1).

Taking the swab or aspirate from the infant patient requires patience and experience; consequently, there are significant differences among pediatricians following the same basic diagnostic procedure. Similarly, the aspirate or washing should be taken from the posterior nasopharynx.

Transport of samples. Direct plating of specimens onto selective media in the pediatrician's office is reported to be the method of choice (18, 31, 32). However, direct plating techniques require some amount of technical skill. If direct plating is not possible, a transport medium must be used. Nonnutritive antibiotic-free media such as Stuart medium or Ames medium should be supplemented with charcoal, but are only feasible if the mean transport time does not exceed 24 h. As shown in Table 1, such media also allow for the frequent detection of other possibly pathogenic bacteria from swabs originally intended for culture of *B. pertussis* or *B. parapertussis.* If the transport time is expected to exceed 24 h, a selective enrichment medium should be used; Regan-Lowe medium (33), a half-strength charcoal agar with horse blood and cephalexin, is superior to other formulations. Regan-Lowe medium allows for the selective enrichment of *B. pertussis* while suppressing the growth of normal NP flora (13, 33). The temperature of the transport system remains a point of debate: while ambient temperatures have been reported to suppress growth of *B. pertussis,* preincubation at 35°C may result in the overgrowth of NP flora (29; J. E. Hoppe, Letter, J. Clin. Microbiol. **27**:595; W. E. Morrill, Author reply, J. Clin. Microbiol. **27**:595-596). On the other hand, refrigeration of the transport system reduced the number of viable bacteria.

Isolation of *B. pertussis* and *B. parapertussis*. Several studies have convincingly demonstrated that charcoal horse blood (CH) agar is superior to Bordet-Gengou (BG) agar for the isolation of *Bordetellae* from clinical material (7, 19, 23, 29, 30, 33, 34, 36, 39). Some authors have advocated the combined use of CH and BG media

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* Presenting author.

TABLE 1. Microbiological findings in NP swabs from 1,260 patients with clinical symptoms of whooping cough

Culture	*n*	%
B. pertussis (pure culture)	489	39
B. pertussis with *S. aureus*	30	2
B. pertussis with pneumococci	29	2
B. pertussis with *H. influenzae*	19	2
B. parapertussis	54	4
Pneumococci	144	11
S. aureus	69	5
H. influenzae	67	5
ß-hemolytic streptococci	20	2
Other possibly pathogenic bacteria	9	1

(2, 23; F. Chan, E. Rossier, A. M. R. McKenzie, and A. Comos, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C260, p. 335). For routine use, however, BG has the disadvantage of a very short shelf-life; also, some commercially available BG preparations are significantly less sensitive than self-made medium (39). Other media, such as buffered charcoal yeast extract agar or cyclodextrin solid medium, have been described for the isolation of *Bordetellae* from clinical samples. Both media were found less sensitive than CH medium (12, 17, 29, 39), although cyclodextrin solid medium was reported to be as sensitive as BG medium (3).

CH agar is usually supplemented with horse blood, which has been found to be superior to either blood-free medium or blood from sheep or humans (15, 17). Human blood, however, is serviceable when no other source is available.

Normal NP flora is inhibited mostly by cephalexin, which has been found to be superior to penicillin (34, 36), methicillin (34), lincomycin (17), and cefsulodin (16). The use of both selective and nonselective media has

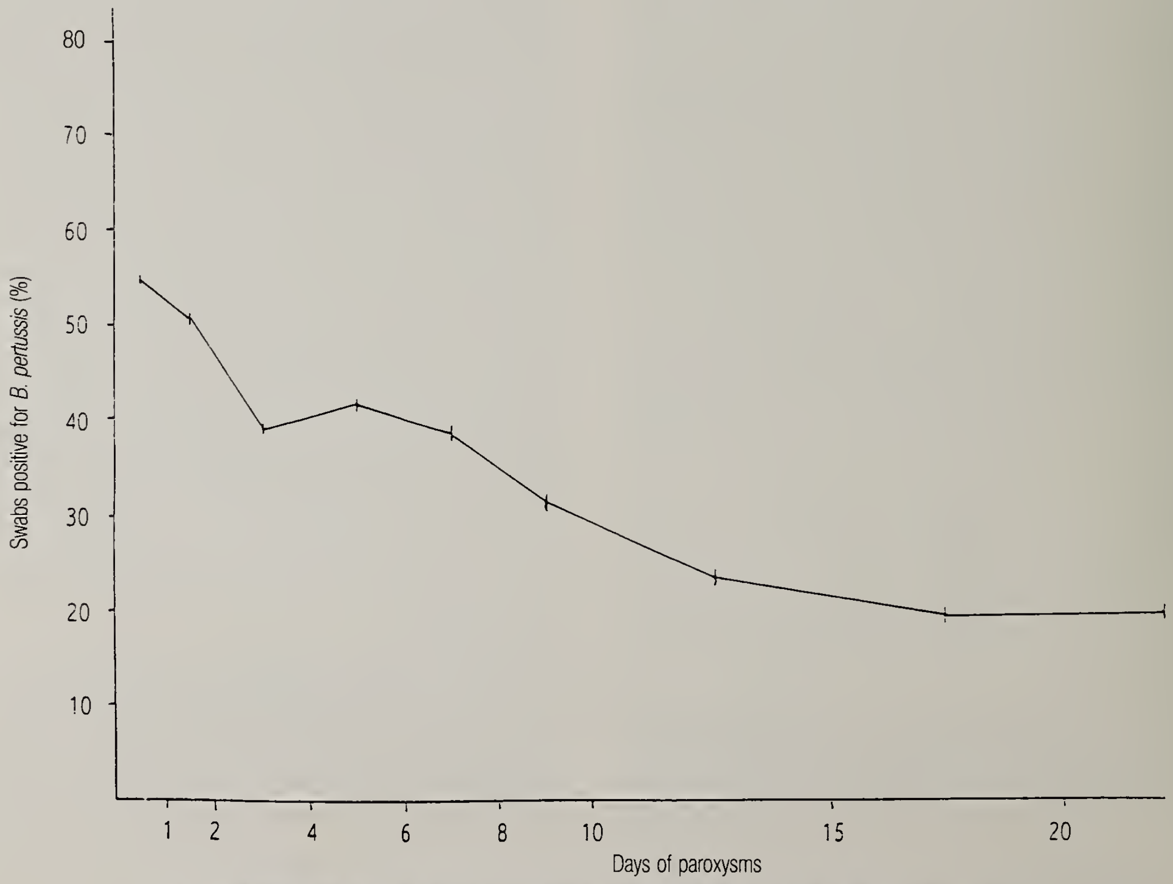

FIG.1. Isolation rate of *B. pertussis* from NP swabs related to the length of time of paroxysmal coughing.

TABLE 2. Age dependency of bacteriological findings in NP swabs from 956 children with clinical symptoms of whooping cough

Culture	Group I[a] (%)	Group II[b] (%)	Group III[c] (%)	Group IV[d] (%)
B. pertussis	47	50	45	40
B. parapertussis	3	7	4	5
H. influenzae	16	13	7	5
Pneumococci	11	9	11	14
S. aureus	6	7	6	3
Others	2	2	3	4

[a] Less than 6 months old (*n* = 62).
[b] 6 to 12 months old (*n* = 90).
[c] 1 year to 6 years old (*n* = 706).
[d] 6 years to 18 years old (*n* = 100).

been suggested (5), as some strains of *B. pertussis* are inhibited by cephalexin. However, in testing a total of 233 wild strains from our clinical material, no single cephalexin-sensitive strain was found. The use of other cephalosporins such as ceftibuten or cefetamet has still to be evaluated (14).

Enrichment media. To date, researchers have described only two liquid or semisolid enrichment media: soft charcoal agar (33) and cyclodextrin-supplemented Stainer-Scholte broth (39). Although Regan-Lowe medium increased the isolation of *Bordetellae* by about 6% as compared to CH medium, problems were observed with the overgrowth of cephalexin-resistant NP flora (12). Modified Stainer-Scholte medium, however, allowed for the additional isolation of 12% of *B. pertussis* strains from clinical material that did not primarily grow on CH medium (39). In contrast, only Regan-Lowe medium was effective as an enrichment medium for *B. parapertussis.*

Isolation of *Bordetellae* in relation to disease. Data about the frequency of isolation from clinical material vary tremendously. As noted by Kwantes and co-workers (24), the isolation rate of *Bordetellae* from clinical material is negatively correlated with the duration of disease (4, 35, 38). The relation between isolation of *B. pertussis* and the duration of paroxysmal coughing in our material is demonstrated in Fig. 1. Although more than 54% of swabs were positive for *B. pertussis* at the beginning of the convulsive phase, the rate of positive swabs decreased to 20% in patients who had suffered from paroxysmal coughs for 3 wk or more. However, Fig. 1 also suggests that at least 20% of pertussis patients may still be contagious after the clinical symptoms (i.e., catarrhal and convulsive phase) have lasted for more than 1 month.

Table 2 shows that the isolation of *Bordetellae* from NP swabs did not significantly depend on the age of the patient: between 40 and 50% of swabs were positive in all four age groups. The isolation of *B. parapertussis* followed a similar pattern, whereas *Haemophilus influenzae* was found significantly more often in swabs from infants as compared to older children.

In this study, *Bordetellae* were more often found in swabs taken from girls (*n* = 239) than from boys (*n* = 208), although the disease itself was diagnosed in almost the same frequency. However, the mean duration of paroxysmal symptoms before swabbing was found to be 4.3 days in girls and 7.5 days in boys, offering a possible explanation for the different isolation rates.

As opposed to the findings in infantile whooping cough, *B. pertussis* was isolated in only 11% of the NP swabs taken from 169 adult patients.

Tables 3 and 4 show the clinical symptoms of those patients from whom *B. pertussis* was isolated compared to patients with negative bacteriological results. Surprisingly, it was found that all symptoms were reported in

TABLE 3. Clinical symptoms and hematological findings in whooping cough patients with cultural isolation of *B. pertussis*[a]

Clinical finding	*n*	%
Mean duration of paroxysms	6 days	
Nonparoxysmal cough	225	68
Mean duration	12 days	
Whooping	128	39
Vomiting	198	60
Nocturnal coughs	137	41
Fever	45	14
Mean temperature	38.5° C	
Contact anamnesis	189	57
Leukocytes/µl (*n* = 166)	13,200 ± 4,900	
Lymphocytes rel% (*n* = 54)		61
Neutrophils rel% (*n* = 54)		34

[a] *n* = 331.

TABLE 4. Clinical symptoms and hematological findings in whooping cough patients without cultural isolation of *B. pertussis*[a]

Clinical finding	n	%
Mean duration of paroxsyms	6 days	
Nonparoxysmal cough	203	68
Mean duration	12 days	
Whooping	117	39
Vomiting	178	60
Nocturnal coughs	129	43
Fever	40	13
Mean temperature	38.5°C	
Contact anamnesis	176	59
Leukocytes/µl ($n = 155$)	13,600 ± 5,200	
Lymphocytes rel% ($n = 42$)		61
Neutrophils rel% ($n = 42$)		33

[a] $n = 298$.

very similar frequencies among the two groups; it also holds true for the absolute number of white blood cells and the relative numbers of polymorphs and lymphocytes.

Thus, the data indicate that the cultural isolation of *B. pertussis* from the NP tuft did not depend only on the severity of symptoms, but is also influenced by other factors yet unknown.

In addition to these above-described cases with typical symptoms, *B. pertussis* was also isolated from a total of 189 patients with longer lasting non-pertussis form coughs, indicating that nontypical cases may account for at least 20% of all cases in endemic areas.

Detection of structural components of *B. pertussis* in clinical specimens. Various alternative methods have emerged in recent years for the detection of structural components of *B. pertussis;* some of these methods have also been tested for use with clinical material.

Pertussis toxin has been detected in NP aspirates by either dot blot immunoassay or clustering of Chinese hamster ovary cell cultures (6, 10). Whereas the specificity of both methods as compared to culture was found to be between 88 and 98%, sensitivity ranged from 45 to 88%.

The enzymatic activity of adenylate cyclase toxin was also used for direct detection of the bacteria (28, 37). The analysis of 250 wild strains of *B. pertussis* showed that all strains produced measurable activities of the enzyme. In clinical specimens, adenylate cyclase activity was detected with a sensitivity of between 45% (NP swabs) and 71% (NP aspirates) as compared to culture; specifity was between 97 and 100%.

Structural components of *B. pertussis* were also measured after preceding culture of the bacteria in different media to reduce the time to diagnosis. Detecting pertussis toxin by Chinese hamster ovary cell clustering reduced the time to diagnosis by more than 1 day (10). Similarly, measuring adenylate cyclase activity in modified Stainer-Scholte medium detected 92% of *B. pertussis* after 72 h of culture (37). Another attempt was made with a colony-blot assay employing antibodies to either filamentous hemagglutinin or lippopolysaccharide (9); this also speeded up the detection of different strains by at least 1 day.

Probing for *Bordetella* DNA represents another approach for detecting the presence of the bacteria. A probe for a repetitive sequence of *B. pertussis* has been evaluated with clinical material (27, 28); sensitivities vary between 43 and 86% for either NP swabs or aspirates. Specificities as compared to culture were 98 and 82%, respectively, for NP swabs and aspirates. The use of polymerase reactions may increase the probe's sensitivity of nucleic acid detection. Polymerase reactions for the detection of *B. pertussis* in clinical specimens have been developed (21; A. Bollen, S. Hovard, C. Hackel, P. Jacobs, A. Herzog, A. Delem, J. Petre, and F. Ambrose; J. R. Oksenberg and L. Steinman), and preliminary results on detecting the bacterial DNA in clinical specimens look promising.

During the last decade, the isolation of *Bordetellae* from clinical material was improved by various attempts to compare and standardize different kinds of clinical specimens, transport systems, and culture media. Although, an array of alternatives to conventional culture have emerged, the sensitivity and specificity of these methods in diagnosing whooping cough or *Bordetella* infections have yet to be defined. Comparative studies in endemic and nonendemic areas are needed to select for an optimal combination of direct detection methods for *B. pertussis* and *B. parapertussis* from clinical material.

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DISCUSSION

DR. PARKER: Let me make a couple of comments on some work that was done partially by myself and partially by Beverly Paine in the Missouri State Health Department. We generally have our pertussis outbreaks in the summer and it is very hot in Missouri and Beverly Paine has gone to using a refrigerator temperature for transport in Regan-Lowe and finds this much superior to ambient. It may not apply in Germany if you have cold summers, I do not know.

DR. WIRSING VON KÖNIG: Yes, this is one of the reasons why we could use ambient temperature. Except for this summer, we have rather cool summers and the peak incidence in our country is between late October and mid-January.

DR. PARKER: If cephalexin is ordered directly from a chemical company, we have shown that it requires a level of 10 micrograms per mil rather than 40. Positives go up six-fold on incoming specimens in that case. When you test after you have cultured them already, of course they will grow in cephalexin. But when you have a few organisms in the culture surviving under adverse conditions, so that is the other thing. The last comment, Beverly tells me that the state of Tennessee and now Missouri are recovering organisms about 25 percent of the time. They get a new 25 percent recovery if they use, in addition to Regan-Lowe, just saline which has been capped soon after autoclaving. So saline alone if kept cold is a good transport medium. It is not as good as Regan-Lowe, but used in conjunction it verifies your data.

DR. WIRSING VON KÖNIG: We never tried to use saline and what you said about cephalexin. However, our strains which we tested for cephalexin resistance and sensitivity, were all isolated from primary cephalexin-free medium. However, I would agree that 40 micrograms, I think this is historically explainable that we should use 40 micrograms and we are actually comparing these and we should come down to 20 or 10 micrograms.

DR. AOYAMA: Do you use Regan-Lowe transport medium?

DR. WIRSING VON KÖNIG: We do use Regan-Lowe; when longer transport times are anticipated we use Ames medium with charcoal.

DR. AOYAMA: The number of organisms present is important. Immunized children secrete small numbers of organisms but unimmunized children secrete lots of organisms. I prefer Ames transport medium with charcoal and find that it is better. I have current data which shows Ames transport medium works well for more than 24 hours.

DR. WIRSING VON KÖNIG: I am quite sure it may work over 24 hours but we encountered some problems with overgrowth by nasopharyngeal flora.

DR. AOYAMA: Yes, but if you use charcoal agar with 40 microgram cephalexin, that is okay.

DR. WIRSING VON KÖNIG: But would you see a definitive correlation between the number of bacteria irrespective of transportation time and irrespective of how the swab has been taken? We always have been a little bit unwilling to quantitate the bacteria in swabs.

DR. AOYAMA: Yes, if you transport for a long time, the bacteria die. And a related issue, the quality and usefullness of Bordet-Gengou medium depends on the manufacturer. Some manufacturers make good media, others do not.

DR. WIRSING VON KÖNIG: Well, we do not have so many manufacturers in Germany.

Serodiagnosis of Pertussis

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Pertussis is difficult to diagnose with currently available technology. Diagnosis is based on detection of either *Bordetella pertussis* or its products or specific host immune responses. These alternatives are complementary and improve the rate of case diagnosis. During the catarrhal stage, before symptoms become characteristic, the organism is most easily detected. The traditional procedures are culture and immunofluorescence. Culture is slow and suffers from low sensitivity, especially in laboratories that do not perform the procedure regularly. With optimal sampling time, experienced laboratories can isolate the organism in 50 to 70% of cases (38, Wirsing von König, this volume), but the results are not available for 3 to 5 days. More commonly, sampling time is not optimal, and isolation rates often drop below 20%. Immunofluorescence is more rapid, but also suffers from low sensitivity and requires subjective evaluation (10, 17, 19, 38). Specificity is an additional problem with the commonly available polyclonal reagents, but, as indicated in recent studies (Bromberg, K., C. Gullans, G. Tannis, Z. M. Li, M. J. Brennan, and C. R. Manclark. Sixth Internat. Symp. on Pert. Abstracts. 1990. No. 65, p. 201-203), newer monoclonal reagents may allow immunofluorescence to become more useful clinically. Several diagnostic developments are on the horizon to improve the ability to detect *B. pertussis* in the nasopharynx, including polymerase chain reaction and nucleic acid probe technology (12, 20) and antigen detection procedures (11).

The fact remains, however, that the number of organisms in the respiratory tract decreases at the same time that symptoms are becoming characteristic (21). This is also the time when specific immune responses, including cellular, mucosal, and serologic, appear in the host. Although host cellular responses to antigens of *B. pertussis* can be measured (6, 52, Blumberg et al., this volume), the technology is not easily adapted to clinical laboratory use. Recent papers have discussed the possibility of using mucosal antibody responses for diagnosis (4, 13, 37, 45, 59, Thomas et al., this volume), but there are insufficient data to evaluate this approach fully. This report addresses the possibilities and limitations of using serology to diagnose pertussis, focusing on (i) the major obstacles to the general application of serodiagnosis, (ii) procedural and methodological issues, and (iii) assay selection. Recommendations are presented for assay procedures to be used in different clinical settings.

OBSTACLES TO THE CLINICAL APPLICATION OF SERODIAGNOSIS

A wide variety of serologic procedures have been explored during the 86 years since Bordet and Gengou first isolated *B. pertussis* (24, 26, 27, 29, 40, 53). Among the antibodies measured are complement fixing, bactericidal, precipitating, opsonizing, agglutinating, and toxin neutralizing. Although these procedures were of value in understanding the immune response to infection, all suffered from technical problems that limited their diagnostic use in a clinical laboratory. Enzyme immunoassay procedures, however, do have the potential to be adapted to a routine laboratory setting, and will be emphasized here. Enzyme-linked immunosorbent assay (ELISA) technology has been used extensively to either examine host immune responses or diagnose infection (29, 53). Despite the fact that essentially all authors concluded that ELISA was useful for diagnosis, it is still used primarily as a research tool and is used on a routine basis in only a few clinical laboratories.

The ideal solution for serodiagnosis is a simple, nonquantitative test kit in which an indicator turns color if a patient has pertussis. Unfortunately, this solution is not possible with current technology. The biggest difficulty is that the serologic tests must be accurate and quantitative, because there are few individuals in the world who have not been exposed to *B. pertussis* either by vaccination or infection. Therefore, any individual may potentially have circulating antibodies to a variety of *B. pertussis* antigens. To date, no immune response has been identified that can reliably distinguish infection from vaccination or differentiate a recent infection from an earlier one. Because absolute differences in antibody concentrations have not been observed, serodiagnosis depends upon quantitative differences and can be based on the observation of either antibody concentrations above diagnostic cut-off values or significant changes between paired sera. Given the requirement for accurate quantitation, the tests are unlikely to be used routinely in a physician's office; however, there is no reason that they should not be available in commercial laboratories, tertiary hospitals, and state health departments.

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* Presenting author.

Additional obstacles to the general application of serodiagnosis are the unavailability of highly purified antigens, the lack of standardization, and the employment by many labs (including our own) of procedures that are labor intensive and require complicated mathematical analysis. Even though procedures must be quantitative, they can and must be simplified if they are to find general application in clinical laboratories.

METHODOLOGICAL CONSIDERATIONS

As discussed above, serologic diagnosis can be based on either the demonstration of significant changes in specific antibodies in paired specimens or the observation of antibody concentrations above established cut-off values in single samples. Both of these approaches need technical refinements. Diagnosis based on the demonstration of significant changes is certainly the most reliable approach. However, each laboratory must define, on the basis of the methodology selected, the changes that can be detected confidently and reliably. When defining diagnostically significant changes, both technical and biological issues must be considered. To assess the technical reproducibility, coefficients of variation can be established by repetitive testing of a single sample. Recent data indicate that an experienced worker using optimal procedures could detect 50% changes for sera containing antibodies within the working range of the assay (Meade, B. D., H. O. Hallander, T. A. Romani, L. Mardin, C. B. Swartz, M. Ljungman, B. Reizentstein, R. Mollby, W. C. Blackwelder, and C. R. Manclark. Sixth Internat. Symp. on Pert. Abstracts. 1990. No. 69, p. 207.) Reproducibility of the procedure must be measured for samples falling in different parts of the response curve; coefficients of variation certainly increase near the extremes of detection. Samples with very high antibody concentrations can be diluted to fall within the optimal detection range, however this is not possible for samples with low concentrations. For such samples, a greater percentage change is required for significance.

Issues other than the technical reproducibility of the assay must also be considered. There are some viral, parasitic, autoimmune, and inflammatory diseases in which nonspecific increases in antibody levels occur because of polyclonal activation (44), although few of these diseases are likely to be confused clinically with pertussis. Because symptoms in many infants, adults, and immunocompromised patients with pertussis are not characteristic (9, 25, 32, 34, 46), it is possible that individuals with nonspecific polyclonal responses could be diagnosed incorrectly as cases of pertussis.

Although serodiagnosis based on the demonstration of significant antibody changes is more reliable, this is not the situation most commonly seen clinically. The requirement for an interval of at least 3 weeks between paired samples dictates that serology be used for retrospective diagnosis. Additionally, in many cases, antibodies have reached a plateau level by the time the patient is first seen, and significant decreases are not observed for several weeks (8, 46). Therefore, diagnosis based on observation of elevated levels is often the most practical for patient care. Such a procedure is inherently less sensitive, but a number of studies have shown that a reasonable balance of sensitivity and specificity can be achieved (19, 25, 31, 39, 42, 46, 51). What is needed now is a definition of diagnostic levels: these levels must be based on a consideration of age, vaccination status, and the state of health of the patient (e.g., presence of other acute or chronic illnesses and immunological status), as well as prevalence of pertussis infection in the community. A set of diagnostic criteria can be developed based on antigen and isotype responses. For example, a diagnosis of pertussis can be considered likely if a patient has one test result more than 3 standard deviations above the control group mean in one assay or more than two standard deviations above the mean in two or more tests. Such criteria would have less than 100% sensitivity and specificity, but would be a useful adjunct to the procedures currently available.

ASSAY AND ANTIGEN SELECTION

Two approaches have been used when selecting antigens for investigational use. One approach employs pure components as the coating antigens in ELISA (1, 3, 9, 15, 17, 18, 19, 25, 30, 35, 39, 43, 46, 47, 48, 50, 54, 55, 56, 57, 58), and the other uses whole bacteria or extracts (5, 8, 22, 23, 31, 32, 36, 42, 51). The first two proteins of *B.*

pertussis purified in reasonable quantity were pertussis toxin (PT) and filamentous hemagglutinin (FHA); it is therefore not surprising that these were the first two antigens studied in ELISA. More recently, other antigens have been purified and studied, including pertactin (69-kDa outer membrane protein) (45, 49, Romani, T. A., A. Deforest, and B. D. Meade, Abstracts Ann. Meet. Amer. Soc. Microbiol. 1990. No. B-140), fimbriae (50), porin (Hannah, J., Z.-M Li, M. Brennan, A. Karpas, P. Probst, J. Kenimer, and C. Manclark. Ann. Meet. Amer. Soc. Microbiol. 1990. No. B-143), lipooligosaccharide (LOS) (14, 45, 54), adenylate cyclase toxin (Arciniega, J. L., E. L. Hewlett, M. C. Gray, A. Deforest, S. G. F. Wassilak, I. M. Onorato, C. R. Manclark, and D. L. Burns. Sixth Internat. Symp. on Pert. Abstracts. 1990. 39, p. 156-157), and chaperonin (Arciniega, J., F. Johnson, E. Hewlett, A. Deforest, M. Endoh, and C. Manclark. Ann. Meet. Amer. Soc. Microbiol. 1990. No. B-145). It is interesting to note that not only were PT and FHA the first antigens to be tested, they still appear to be the most valuable for serodiagnosis. For all purified proteins, except PT, the related species *B. pertussis* and *B. bronchiseptica*, produce proteins that cross-react immunologically with the corresponding protein of *B. pertussis*. Antibody responses to PT therefore, are the only responses that can reliably distinguish a *B. pertussis* infection from and infection caused by one of the other *Bordetella* species.

Investigators who have measured isotype-specific responses to these antigens have concluded that the most useful assays, in approximate order of relative value, are immunoglobulin G (IgG) anti-PT (G-PT), IgG anti-FHA (G-FHA), IgA anti-FHA (A-FHA), and IgA anti-PT (A-PT) (15, 19,25, 39, 46, 47). These conclusions are based on the observation that over 90% of infected individuals produce G-PT antibodies, 80 to 90% produce G-FHA, 50 to 60% produce A-FHA, and 40 to 50% produce A-PT (46, 47). There is less agreement on the value of measuring IgM responses to PT and FHA. Our studies are consistent with published reports which concluded that the IgM anti-PT (M-PT) and IgM anti-FHA (M-FHA) assays offered little diagnostic value because the responses appeared in a minority of cases, and, even when present, were almost always accompanied by an IgG response (15, 25, 46). Other investigators have found M-PT and M-FHA responses to be helpful (19, 45, 50). It is not clear if these differences are due to technical variations among assay procedures or to immunological differences in the study populations. The reasons for these differences should be explored, but each laboratory can determine on the basis of preliminary studies if the IgM assays have sufficient value to merit inclusion in the assay repertoire. In our experience, a set of reasonable diagnostic criteria can be based on measurement of the four antibodies, G-PT, G-FHA, A-PT, and A-FHA.

There are some special considerations when applying these tests to sera from very young infants. Because of immunological immaturity, the IgA responses in particular appear to be lower and more delayed in this population (Morgan, C., E. Pineda, D. Blumberg, K. Kewis, L. Ross, and J. Cherry. Abstr. Intersci. Conf. Antimicrobial Agents and Chemotherapy. 1989. 257). Infants can make an IgG response to PT and FHA, but care must be exercised to distinguish between infant and maternally derived IgG antibodies.

The prospect of universal immunization with acellular vaccines likely to contain PT and FHA presents a dilemma. Antibody responses to PT and FHA are generally higher in individuals receiving acellular vaccines than those who receive whole-cell vaccines (2, 7). Diagnostic levels would therefore need to be revised, and the value of these assays in serodiagnosis would decrease. The problem would be accentuated if, as is likely, acellular vaccines receive widespread adult use. A high priority, therefore, is the development of diagnostic assays based on antigens other than PT and FHA.

It has been suggested that measurement of either IgG subclass responses or IgA responses could differentiate vaccination from infection. The two studies (16, 58) that have investigated the IgG subclass response to pertussis vaccination or infection are somewhat contradictory, and future study is warranted. Immunoglobulin A responses occur more frequently in infection than vaccination (29, 36), and therefore are helpful for diagnosis when observed. However, it is our experience that only about 50% of infected individuals make an IgA response to PT and FHA; therefore, criteria based solely on these assays would have low sensitivity.

An antigen worthy of additional study is LOS (endotoxin), because it is unlikely that LOS will be included as an immunogen in the next generation of pertussis vaccines. There is little agreement among published reports on the response to LOS in infected individuals (14, 45, 54). Among culture-confirmed cases, we observe an IgM response commonly, an IgG response occasionally, and an IgA response rarely. Others have reported that a high percentage of patients produce an IgA anti-LOS response (14, 54). Granström et al. report that 70 to 80% of previously unimmunized pertussis patients seen at the Karolinska Hospital in Stockholm produce an IgG response to LOS (14). We have shared sera and reagents with Dr. Granström in an attempt to resolve this difference and determine if LOS assays can serve as a complement to PT and FHA assays. Before LOS antibody assays are used extensively in diagnosis, however, additional work must be done to define the specificity of the response. In particular, it must be determined if antibodies to the endotoxin of other species cross-react with the LOS of *B. pertussis*. In several children, we observed an increase in IgM antibodies that reacted with *B. pertussis* LOS was observed in the absence of known exposure (data not shown).

Clearly, other assays need to be identified that can reliably distinguish infection and vaccination. Antibody responses to other antigens have been measured. Among these antigens are outer membrane proteins including pertactin and porin, fimbriae, adenylate cyclase toxin, and the *B. pertussis* chaperonin ("GroEL-like"). Although many infected individuals make an antibody response to these components, a lower percentage of individuals respond to these than to PT and FHA. One possibility was suggested by the work of Arciniega et al. in which the authors observed that five of five infected individuals but zero of nine vaccinated children reacted to a 29-kDa protein band in an immunoblot (Arciniega, J., F. Johnson, E. Hewlett, A. Deforest, M. Endoh, and C. Manclark. Ann. Meet. Amer. Soc. Microbiol. 1990. No. B-145). Although immunoblotting (41, 49) is not readily adapted to routine diagnostics, it could be used as a confirmatory test on sera that had uncertain results in the ELISA assays.

Although we have favored the use of purified antigens in ELISA because they can be more readily reproduced and standardized, a critical reading of the literature suggests that assays which employ non-purified antigens and extracts should also be considered. These assays appear to be somewhat less sensitive than those which use purified PT and FHA, but have comparable specificity. An extract based on published procedures (4, 33, 36), possibly treated to deplete the coating preparation of PT, FHA, and LOS, may provide an alternative approach for diagnosis until new purified antigens are identified. The value of such extracts would be enhanced if produced in a standardized method and characterized adequately.

Two non-ELISA assays that have been widely studied are the microagglutination assay (29) and the Chinese hamster ovary cell assay for PT-neutralizing antibodies(15). Although very specific, the microagglutination assay appears in most cases to be less sensitive than the ELISA assays. One exception to this generalization, however, came from a study of U.S. college students with persistent cough (Morgan, C., E. Pineda, D. Blumberg, K. Lewis, L. Ross, and J. Cherry, 29th Interscience Conf. on Microbial Agents and Chemotherapy. 1989. No. 9). Among students in this study with serologic evidence of *B. pertussis* infection, the microagglutination was the assay most likely to be positive. Several studies have demonstrated that the measurement of PT-neutralizing antibodies by the Chinese hamster ovary cell assay can assist in serodiagnosis; however, this is a difficult assay to perform and requires highly trained staff. In addition, it does not appear to add to the information provided by the ELISA for G-PT (15).

CLINICAL APPLICATION OF SERODIAGNOSIS

There has been considerable controversy on the value of serodiagnosis in pertussis. Some of the uncertainty is based on issues already discussed, i.e., the use of different reagents and procedures, the lack of standardization, and the cumbersome technology. Much of the controversy presumably can be traced to the fact that different investigators are using serology in different settings to answer different questions. The three situations in which serodiagnosis has been employed are epidemiological studies, vaccine trials, and patient management. Each situation must be considered separately because both the available samples and the consequences of an incorrect diagnosis are different, and therefore the diagnostic procedures and criteria applied must be different. For each of these applications, the important characteristics and a recommended approach will be defined.

Most investigators have concluded that serodiagnosis is valuable in epidemiological studies to detect cases previously unrecognized because of the difficulties inherent in culture and immunofluorescence (9, 18, 25, 32, 39, 42, 46, 48). Because such studies are usually retrospective, the accuracy of diagnosis for any specific patient is relatively unimportant. What is critical is that criteria be selected for optimal sensitivity and specificity; the conclusions from the study will not be affected, if the investigator, at least on average, places subjects in the correct group. Epidemiologic investigations are usually initiated when most cases have already been infected for several weeks. Because serum pairs are rarely timed appropriately, serological definition of cases must be based on elevated antibody concentrations rather than on significant changes. The assays to detect G-PT, G-FHA, A-PT, and A-FHA should provide adequate information for case definition. If expected distributions in the community are defined by a reasonable control group, sensitivities and specificities in the range of 80 to 90% can be achieved (17, 19, 25, 46). This range is certainly adequate to understand the extent and course of an outbreak.

Different issues must be considered when serology is used to define cases in pertussis vaccine efficacy trials (47,Olin, this volume). Such trials are likely to begin within the next 1 to 2 years, and careful consideration must be given to the benefits and hazards of employing serodiagnosis. In a clinical trial, small changes in the number of cases in any of the vaccine groups can lead to important differences in the estimates of efficacy. It must be recognized that serology can, at best, document infection with *B. pertussis* and that clinical symptoms must also be considered in defining cases. Because the primary goal of vaccination is to prevent disease, not infection, this has led some to discard serodiagnosis in clinical trials. However, to fully evaluate the efficacy of the tested vaccines and to predict the impact of a vaccination program on the community, it is highly advantageous to employ diagnostic procedures with higher sensitivity than culture and to be able to recognize asymptomatic and atypical infection. Because specificity is critical, serodiagnosis in an efficacy trial must be based on the demonstration of significant changes in antibody concentrations. Diagnosis based on the demonstration of high antibody concentrations in a single sample does not, at the present, have the required specificity. It is even more important that the diagnostic procedure not introduce bias among the vaccine groups. In trials currently being considered, a whole-cell vaccine will be compared to one or more acellular vaccines, all of which are likely to contain PT and most of which are likely to contain FHA. The whole-cell vaccine, in addition, induces antibody to a wide variety of antigens including PT and FHA (1, 3). Therefore, it appears that most, if not all, vaccines in the trial will induce antibodies response to PT and FHA, the two most reliable antigens for serodiagnosis. Antigenic exposure among the vaccine groups will not be identical, however. Differential pre-exposure to antigens among the vaccine groups has the potential of introducing bias among the groups, although it is not clear in which direction the bias will occur. It seems possible that an individual previously exposed to an antigen by vaccination would more likely have a significant increase in specific antibody because he or she was primed for a secondary response; however it is also possible that a significant change would be harder to achieve because of a high antibody concentration in the acute sample.

What approach should be taken for such an efficacy trial? First, it must be remembered that diagnosis by all procedures is easier in a clinical trial, because surveillance procedures are in place and community awareness is high. Children enrolled in the trial should be required to report for diagnostic evaluation early, e.g., after 7 days of cough. Such a schedule offers two important advantages: (i) the diagnostic procedures based on culture or detection of organisms are more likely to be positive at 7 days; and (ii) if the acute serum sample is drawn at 7 days, the chance of detecting a significant change in antibody concentrations between acute and convalescent samples is high. The longer the delay in initial patient examination, the lower the chances of detecting infection by any procedure. Our recommendation for serodiagnosis in the trial is to measure isotype-specific responses to PT, FHA, and possibly LOS and a well-characterized membrane preparation.

Until advances are made, serology will have only a limited role in the management of individual patients. The problems for patient management are very similar to those confronted in epidemiological investigations, i.e., that pertussis usually is suspected after it is too late to culture the organism and after antibody concentrations have already increased. As suggested for epidemiological studies, a reasonably sensitive and specific set of diagnostic criteria can be developed based on the four assays, G-PT, G-FHA, A-PT, and A-FHA. For most circumstances and patients, serodiagnosis based on these assays is more useful than culture and would detect 60 to 90% of cases. Patient therapy would, of necessity, be based on clinical symptoms, and it is unlikely that therapy would be discontinued based on a negative serologic test. Increased diagnostic sensitivity offers several benefits. Many atypical and previously unrecognized cases would be detected, and intervention could be implemented earlier and transmission potentially be reduced. In cases of prolonged cough of unknown etiology, the recognition of pertussis would prevent such patients from undergoing more invasive diagnostic procedures.

The ability to confirm a diagnosis would allow a physician to decide whether or not to continue pertussis immunization. In clinical practice, the usefulness of serodiagnosis would be enhanced if physicians obtained serum samples from family members and close contacts, because pertussis is a highly infectious disease, and isolated cases are rare. It is normal for antibody levels to increase in one or more family members, even those that remain asymptomatic (25, 46).

CONCLUSION

Although technological advances will allow more reliable, sensitive, and prolonged detection of *B. pertussis* organisms, practical considerations suggest that the detection of specific immune responses will continue to complement (but never replace) these procedures. However, work must be done to make the procedures easier to perform and interpret. There is still a need for creative investigators to define specific immune responses that can distinguish infection from vaccination, especially as acellular vaccines gain more widespread use.

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DISCUSSION

DR. WIRSING VON KÖNIG: As far as I saw it on one of your first slides, during the catarrhal phase, you had a positive antibody response and a positive serology of between 40 or 50 percent. We did not find this in our population. Would that be relevant?

DR. MEADE: We actually had this discussion earlier and I think that graph showing the percent seropositivity with time I think depends dramatically on whether or not your population would have a prior vaccination history. That was based on data from the US which would probably relate; they are looking at previously immunized individuals and I think the curve would probably be much more delayed if you were looking at previously unimmunized individuals.

DR. WIRSING VON KÖNIG: And would you expect the percentage of detectable bacteria during the incubation phase being as low as 40 percent or higher? I was not aware of any solid data.

DR. MEADE: I was watching your slide very carefully which indicated that it was much higher earlier on, so that is very interesting. That slide maybe should be revised based on some of your data.

Nasal Immunoglobulin A Responses To Pertussis Toxin, Filamentous Hemagglutinin and Agglutinogens 2 and 3 after Infection with *Bordetella pertussis* and Immunization with Whole-Cell Pertussis Vaccine

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Bordetella pertussis is a noninvasive human pathogen which exclusively infects ciliated respiratory epithelium (13). Adhesion by *B. pertussis* to the base of cilia prevents clearance by the mucociliary stream (9) and allows bacterial replication and release of extracellular toxins at the site of infection (4). Pertussis toxin (PT), filamentous hemagglutinin (FHA), and agglutinogens 2 and 3 (Agg 2/3) contribute to the adherence of *B. pertussis* to ciliated respiratory epithelial cells (6, 11, 14, 15). It has been suggested that secretory immunoglobulin A (IgA) to these or other *B. pertussis* antigens is responsible for inhibiting or eradicating infection of the ciliated respiratory epithelium, whereas circulating humoral antibodies provide immunity to the toxins responsible for the symptoms of disease (10).

In the present study, we have measured IgA to PT, FHA, and Agg 2/3 in nasal secretions from patients with pertussis and from healthy 11-month-old infants 6 weeks after their third DTP vaccination, to determine the magnitude of the secretory immune response to infection and vaccination. We also attempted to determine whether the nasal secretory immune response might be used as a diagnostic marker of recent *B. pertussis* infection.

MATERIALS AND METHODS

Patients with pertussis were recruited as previously described (12). Pertussis was diagnosed in the presence of a paroxysmal cough lasting more than 2 weeks, associated with a typical whoop, vomiting, or apneic episodes (2). Pernasal swabs cultured on charcoal agar supplemented with 10% sheep blood and 40 µg of cephalexin per ml were used to isolate *B. pertussis* from patients with pertussis.

Nasal mucus was collected from patients with pertussis by gently rotating a calcium-alginate-tipped swab on a flexible wire shaft (Transwab Medical Wire & Equipment Co.) against the nasal turbinates and septum approximately 1 to 2 cm proximal to the anterior nares. The nasal-mucus-bearing tip of the swab was then amputated into an airtight polyethylene tube containing 2 ml of transport medium and stored at 4°C until assayed. Serial samples from each subject were stored and assayed in parallel.

Nasal mucus was collected by a similar technique from infants attending vaccination clinics in Hertfordshire, England, who had no history of either paroxysmal cough or exposure to pertussis. A cotton-tipped swab was first moistened in sterile water and then rotated within the anterior nares. The tip of the swab was broken off into a tube containing transport medium, stored, and then assayed in a manner identical to that used for the swabs from the patients with pertussis. Samples were collected from 10-month-old infants 6 weeks after their third immunization with DTP vaccine (Wellcome Trivax, Beckenham, England).

Total IgA and specific IgA to FHA, PT, and Agg 2/3 in nasal mucus were determined by an enzyme-linked immunosorbent assay method as previously described (1). For nasal mucus samples with a total IgA concentration greater than 1 µg/ml, titers of specific IgA were normalized on the basis of total IgA concentration to correct for differences in the volume of nasal mucus collected. Re-

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TABLE 1. Mean $\log_{10}$ (SD) titers of IgA to FHA, PT, and Agg 2/3 in nasal mucus samples

Antibody response	Days from onset of cough: 8-14 days ($n = 13$)	43-84 days ($n = 14$)	P
IgA to FHA	2.36 (0.57)	3.26 (0.90)	0.037
IgA to PT	2.10 (0.58)	2.64 (0.70)	0.133
IgA to Agg 2/3	2.00 (0.40)	3.19 (1.04)	0.003

Samples were collected from patients with culture-positive pertussis at the onset of symptoms and during convalescence. IgA titers were normalized based on total IgA concentration.

sults from samples with a total IgA concentration of less than 1 µg/ml were discarded.

Differences between population means were tested by Student *t* test.

RESULTS

Nasal mucus samples ($n = 258$) were collected between July 1985 and July 1987 from 114 patients with pertussis; patient ages ranged between 1 month and 49 years. The characteristics of these subjects and their serum IgG, IgA, and IgM responses to FHA, PT, and Agg 2/3 have been described previously (12). In brief, episodes of whooping, vomiting, apnea, and cyanosis occurred in 63%, 82%, 66%, and 29% of patients, respectively. Further, 63% of the patients were admitted to the hospital because of their illness. *B. pertussis* was isolated from 30/91 (33%) patients who had a postnasal swab collected within 42 days of the onset of cough. *B. pertussis* was isolated from 7/23, 12/29, 6/18, 4/13, 1/5, and 0/3 subjects who had a postnasal swab collected in weeks 1 to 6 of illness, respectively.

Mean titers of IgA to FHA, PT, and Agg 2/3 in nasal secretions from the 30 patients with culture-positive pertussis rose from approximately $\log_{10}2$ at the onset of symptoms to peak at approximately $\log_{10}3$ between 6 and 12 weeks later (Table 1). Overall mean titers of IgA to FHA, PT, and Agg 2/3 in 84 patients from whom *B. pertussis* was not isolated (16 of whom did not have pernasal swabs collected) were almost identical to those in patients with culture-positive pertussis. However, in samples collected between 8 and 14 days after the onset of cough, mean titers of IgA to FHA, PT, and Agg 2/3 were significantly higher ($P < 0.05$, $P < 0.05$, $P = 0.001$, respectively) in 17 patients with culture-negative pertussis than in 13 patients with culture-positive pertussis. There were no significant differences in mean titers of IgA to FHA, PT, and Agg 2/3 between culture-positive and culture-negative patients for all other time intervals studied (0 to 7, 15 to 21, 22 to 28, 29 to 42, 43 to 84, and >84 days after the onset of cough).

Because the differences in titers of IgA between culture-positive and culture-negative patients for samples collected between 8 and 14 days after the onset of cough might have been due to differences in subject ages in these two groups, the comparison was repeated for subjects aged less than 11 years. Although mean titers of IgA to FHA in 13 patients with culture-positive pertussis (mean age = 1.7 yr, SD = 2.1 yr) and 16 patients with culture-negative pertussis (mean age = 1.4 yr, SD = 2.0 yr) were not significantly different, the mean titers of IgA to PT and to Agg 2/3 were significantly higher in the culture-negative patients ($P < 0.05$ and $P < 0.01$, respectively).

Titers of IgA to FHA, PT, and Agg 2/3 in nasal mucus collected between 28 and 84 days after the onset of cough from 20 patients with pertussis, aged less than 1 year old, were significantly higher ($P \leq 0.0001$) than those in nasal mucus collected from 42 healthy 11-month-old infants 6 weeks after their third DTP vaccination (Fig. 1).

DISCUSSION

Mean titers of IgA antibodies to FHA, PT, and Agg 2/3 in nasal secretions from patients with pertussis were found to increase over the period to 12 weeks after the onset of cough. By this time most subjects had detectable antibodies, although the level varied considerably between subjects. This is the first study in which antibodies to the major fimbrial antigens of *B. pertussis* have been shown to be induced in the upper respiratory tract where they have the potential to inhibit fimbriae-mediated adherence. Although the nasal mucosal responses were modest, they were of the same order, showed the same overall progression, and persisted as long as the serum antibody responses (12). Other studies of nasopharyngeal antibody responses to *B. pertussis* antigens have not shown large rises in antibody titers with duration of illness (7, 8). The protective significance of these apparently modest secretory responses to *B. pertussis* antigens is unknown. Also, they may not be typical of the levels of response at other significant sites of mucosal immunity such as the trachea or bronchi.

We found that titers of antibodies in nasal mucus collected during the second week of cough were significantly lower in culture-positive patients than in culture-

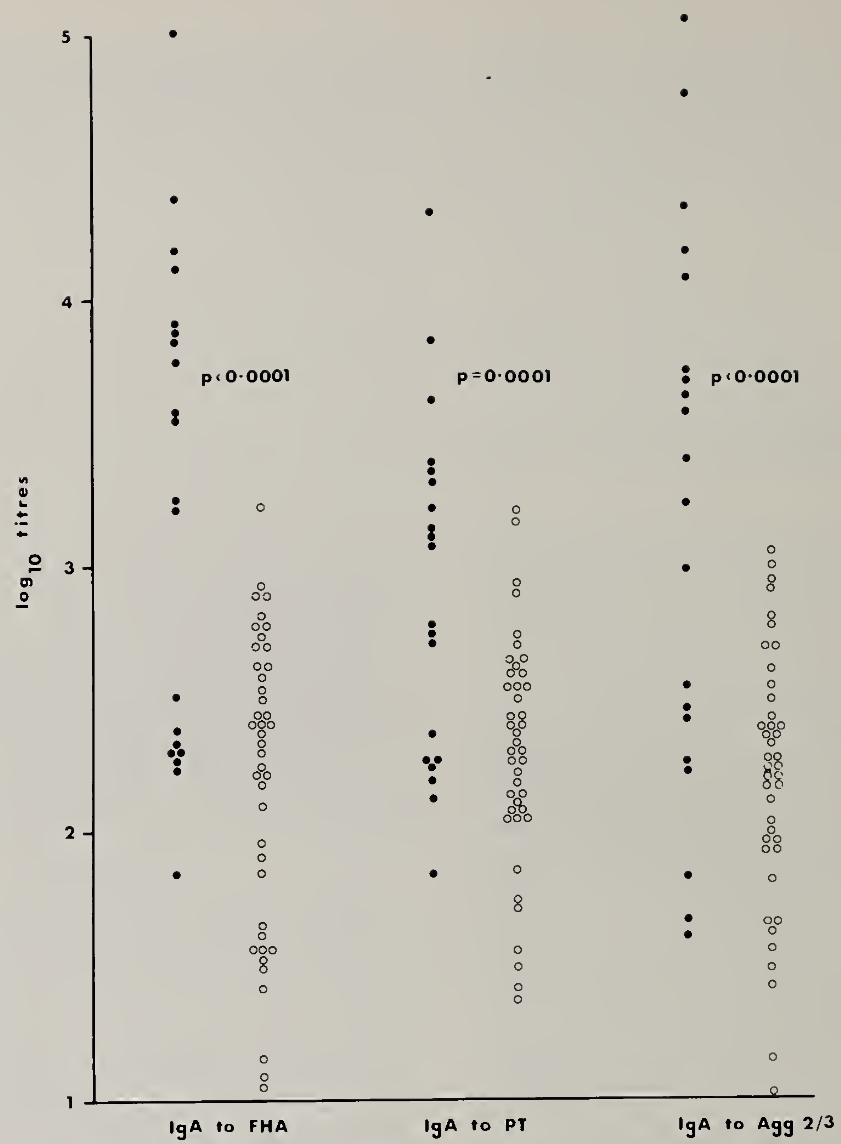

FIG. 1. Log_{10} titers of IgA to FHA, PT, and Agg 2/3 in nasal mucus from 20 infants aged less than 1 year with pertussis (●), and from 42 11-month-old healthy infants 6 weeks after their third DTP vaccination (○).

negative patients, suggesting that secretory responses may contribute to the eradication of *B. pertussis* infection. This finding should be interpreted with caution as the rate of isolation of *B. pertussis* in the patients studied remained at about 30% throughout the first 4 weeks of cough.

Other workers (3, 5, 7) have suggested that detection of *B. pertussis*-specific IgA in respiratory secretions might be a useful adjunct to culture in the diagnosis of pertussis. We found that mean titers of specific IgA in nasal mucus from infants 4 to 12 weeks after the onset of cough were significantly higher than those in vaccinated infants with no history of pertussis. Although titers in these two groups of patients demonstrated considerable overlap, the results suggest that the finding, in an infant, of a $\log_{10}$ titer greater than 3.3 (for IgA to FHA, PT, or Agg 2/3) is strong retrospective evidence in favor of a diagnosis of pertussis.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Department of Health and Social Security (to M.G.T.).

We thank the subjects and their families for their generous cooperation; the nursing and medical staffs of St. Georges Hospital, Kingston Hospital, Brocklebank Health Centre, and the vaccination clinics of Hertfordshire Health Authority for valuable assistance; Andrew Robinson and Laurie Irons for providing the antigens used in the enzyme-linked immunosorbent assays; Andrew Day and Nichols Hornigold for assisting with enzyme-linked immunosorbent assays measurements; and Valerie Dickinson and Andrew Tickle for data analysis.

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DISCUSSION

DR. MEADE: I have one somewhat technical question which is a point I probably missed. Did you standardize these on the basis of total IgA so these are titers, did you dilute them to constant IgA levels and look at dilutions or how was that done?

DR. THOMAS: We measured total IgA in the specimen that was sent to the laboratory so that is a specimen that contains two milliliters of transport medium with a swab of nasal mucous in it and measured total IgA in that specimen together with specific IgA in that specimen and divided the specific IgA by the total IgA to get a normalized titer. So, we did not adjust the specimen to a constant IgA concentration. We did that mathematically.

DR. MEADE: But the titers you report are titer per milligram IgA?

DR. THOMAS: All these results are titers per milligram IgA.

DR. MEADE: Was the amount of IgA proportionate to total protein or was that considered as an option?

DR. THOMAS: We did not measure total protein. Ted Ashworth actually did the results but I certainly saw no results for total protein. We only measured total IgA.

DR. PETRE: I would like to add one piece of information which in fact relates to the three presentations that we just heard. We are studying the use of nasal aspirates as a diagnostic test for pertussis. This has been done in collaboration with Alex Bollen at the University of Brussels and Professor Ambrosch of Vienna. There was a poster about that. We have used the nasal aspirates to detect secreted IgAs against toxin and FHA and also to detect *Bordetella pertussis* DNA by polymerase chain amplification. The detection of DNA was most sensitive when done on samples obtained within 10 days after the onset of symptoms. The earlier the better, in fact, when the IgA response was appearing much later. So, in fact, better efficiency was obtained by combining the two determinations simultaneously on samples.

DR. WEISS: I think maybe another way of looking at your decline in antibodies in your patients who are culture positive may be not that their responses were not as good but maybe that they have a larger bacterial load and in fact may be using their antibodies more than individuals with a smaller burden.

DR. THOMAS: That is a very good suggestion. The surprising thing was that it was only found in that one time interval. It may reflect the number of specimens that we had available then compared with the surrounding time intervals and finding the trend that was able to achieve statistical significance.

DR. GRANSTRÖM: I found the data very interesting and I just wanted to add a piece of information. Perhaps from some unpublished studies there is a bit of a warning concerning the use the antibody responses for diagnostic purposes. A few years ago we made a pilot study in pertussis patients and compared the antibody levels to IgA levels to PT and FHA to a control group of other infections. This pilot study was very promising and then we went to look at a clinical setting where the main reason why patients suspect they have whooping cough is that they know about an exposure. It turns out that the titers were equally high in the aspirates from those who became patients and those who did not have pertussis confirmed by other methods but have been truly exposed. These were IgA titers which is, of course, very promising for those who believe in the role of secretory antibodies in protection against disease but it was a bit of a disappointment for diagnostic purposes.

DR. THOMAS: We compared titers of IgA to those three antigens in patients with pertussis at various times after the onset of cough with titers of those three antibodies in nasal mucous from family contacts and found no significant difference at any point, rather suggesting to me that either we were not obtaining samples early enough or that nasal antibody is not clearly correlated with protection against development of disease. This emphasizes what I said at the beginning of my talk and that I am more dubious of the benefit of nasal antibody responses than some other people might be.

Epidemiology of Pertussis in Philadelphia

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The introduction and then routine administration of diphtheria-tetanus-pertussis (DTP) vaccine caused a dramatic decrease in the mortality and morbidity of infection due to *B. pertussis* in the United States in the late 1940s and early 1950s. The annual case rate of approximately 110/100,000 population in 1922 to 1948 fell to 0.5/100,000 in 1981 (4, 5, 20). Vaccine efficacy was estimated to be 91% to 94% (3).

However, neither immunity following natural disease nor immunization is complete or lifelong. Pertussis cases continued to be reported in unvaccinated and some vaccinated individuals. In addition, rising concerns about possible serious vaccine-related adverse effects, a perception of diminished risk of infection and poor efficacy of whole cell vaccine, and vaccine shortage led to indifference to and even avoidance of DTP vaccine administration in the early and mid 1980s. Since 1981, the annual incidence of pertussis has risen steadily, with all 50 states reporting cases in recent years (5). In part, this prevalence may be due not necessarily to more cases than in the past, but rather to increased medical awareness of and public interest in the disease, improved methods for diagnosis, and increasing state participation in an active surveillance project that began in 1979 (4). Since 1976, more than five cases of pertussis have been confirmed annually in children whose families seek care at St. Christopher's Hospital for Children (SCHC), a primary and tertiary care medical center in North Philadelphia. Initially, direct fluorescent antibody (DFA) staining of nasopharyngeal secretions was the primary method of diagnosis. Beginning in 1979, culture on Bordet-Gengou agar was added to routine diagnostic studies, use of casamino acid holding medium was introduced, and studies of pertussis serology were begun. Since 1982, DFA testing and culture utilizing Regan-Lowe charcoal agar with cephalexin have been the microbiologic methods constantly employed for diagnosis. This paper reports the epidemiology of pertussis and the comparative usefulness of the diagnostic methods, as well as observations made on the infection and immunity based on clinical and serologic studies.

MATERIALS AND METHODS

Patients with pertussis. SCHC medical records and laboratory data from January 1979 through July 1990 were reviewed for children with symptoms suggestive of pertussis whose diagnosis was confirmed by isolating *B. pertussis* or *B. parapertussis* from nasopharyngeal specimens or by DFA staining of nasopharyngeal secretions. Demographic and clinical data were collected for patients diagnosed since January 1985. Characteristics of patient populations seeking care at SCHC, subdivided by residential postal codes, were extracted from the institution's 1988 statistics.

Clinical and serologic studies. Three clinical studies have used serologic tests. Fisher et al. (7) investigated an outbreak of pertussis in a residential facility for 66 handicapped children and adults. When several residents were noted to have persistent cough in July and early August 1985, pertussis was suspected and confirmed. All residents were then studied and treated with erythromycin for 14 days. Nasopharyngeal specimens and serum were collected at the beginning of the study and 2 months later. Long et al. (14) performed a family study in which each of four children who had symptoms typical of pertussis and positive cultures and their 18 family members were studied for 1 year. Nasopharyngeal specimens and serum were collected from the index cases and their household contacts at the time of diagnosis of the index case and four times subsequently. Finally, Long et al. (13) performed a prospective seroepidemiologic and clinical study. Incidental to a longitudinal study conducted at an urban and a suburban site, 332 15- to 18-month-old toddlers with documented receipt of three doses of DTP vaccine in their first year of life were observed; paired serum samples were collected 2 months apart.

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Microbiologic methods. Nasopharyngeal specimens for culture and DFA testing were collected by advancing a calcium alginate swab through the nares and holding it in the posterior nasopharynx for 15 to 30 s. For subjects enrolled in clinical studies, the swabs were inoculated immediately onto Regan-Lowe charcoal agar with 10% horse blood and 40 µg of cephalexin/ml; for patients seen in routine inpatient and outpatient care areas at SCHC, the swabs were held for no more than 4 h in 1% casamino acid liquid holding medium prior to inoculation. Finally, for specimens sent from other hospitals or laboratories, personnel at referral sites were encouraged to inoculate Regan-Lowe agar and incubate cultures prior to transport. Nasopharyngeal swab specimens were also used to make smears for DFA staining using fluorescein-conjugated rabbit anti-*B. pertussis* and anti-*B. parapertussis* immune globulins (Difco Laboratories, Detroit, Mich.). Cultures were incubated at 35°C in a humid environment with 5% CO_2 and were examined daily for 1 week for growth of typical colonies. Isolates were identified using the DFA staining technique. A DFA test of nasopharyngeal smear or culture smear was considered positive for *B. pertussis* if short rods with a rim of light green fluorescence appeared after staining with the homologous antiserum and if no fluorescence was observed with the heterologous (*B. parapertussis*) antiserum.

Serologic methods and case definition. Serum was stored in aliquots at -70°C and all specimens from an individual were tested simultaneously. Agglutinating antibody was assayed by a microagglutination technique using a suspension of formalin-killed strain 460 *B. pertussis* (15). Pertussis antitoxin was assayed by the in vitro neutralization test using microplate culture of Chinese hamster ovary cells (8). An enzyme-linked immunosorbent assay (ELISA) (25) was used to measure antibodies of immunoglobulin G (IgG) to pertussis toxin (PT-G) and IgG and immunoglobulin A (IgA) to filamentous hemagglutinin (FHA-G, FHA-A).

Purified antigens, controls, replicate testing, standards, and calculation of unitage relative to U.S. Reference Pertussis Antiserum (human) lot 3 (Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md.) were used as previously described (14, 15). ELISA unitage was calculated using the parallel line bioassay method. Antibody was said to be present if the value exceeded 3 standard deviations above the geometric mean value of a negative control population of unimmunized children older than 6 months. Infection due to *B. pertussis* was confirmed by positive culture or DFA test or by serologic evidence of infection. The latter was defined as having at least two positive antibody tests with either a fourfold change in value or a high value. An antibody level was said to be high if it exceeded 3 standard deviations above the geometric mean value of a vaccine control population of immunized children matched for age. Infection was defined as symptomatic if a respiratory illness occurred with coughing of any duration as a notable symptom.

RESULTS

Endemic pertussis. From January 1979 through July 1990, 381 cases of infection due to *B. pertussis* and 9 cases due to *B. parapertussis* were confirmed by culture or DFA testing of children brought to medical attention because of pertussoid symptomatology. The number of cases of *B. pertussis* infection has increased over the 11.5-year period reviewed, without obvious cycling (Fig. 1). The year 1990 has peak occurrence rate with the number of cases during the first 7 months exceeding previous annual rates. Since 1985, at least eight cases

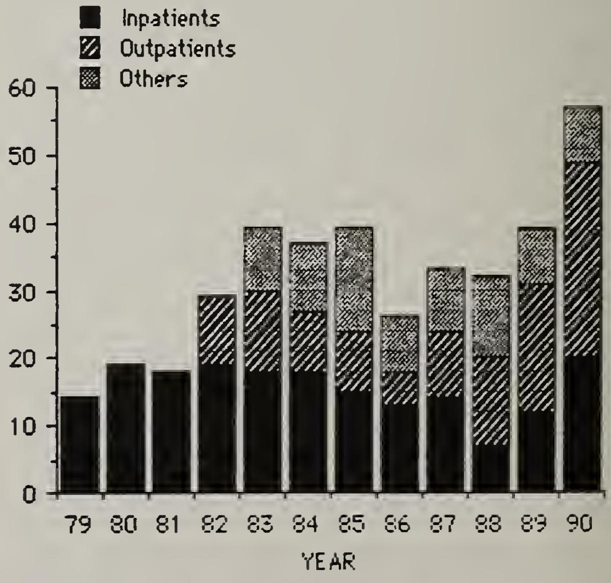

FIG. 1. Distribution of cases of pertussis from January 1979 through July 1990 confirmed by culture or DFA test performed on specimens sent from inpatients and outpatients at SCHC and referred from other hospitals and laboratories.

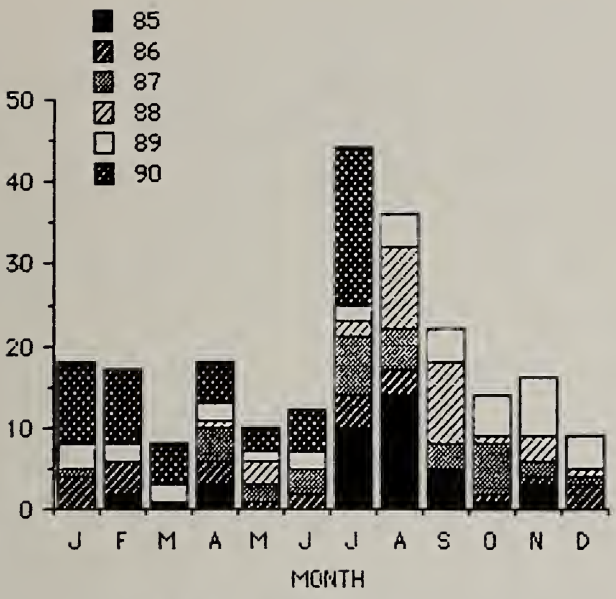

FIG. 2. Distribution of cases of pertussis by month from 1985 through July 1990.

have occurred in each calendar month, with 46 percent of the cases clustered in July, August, and September (Fig. 2). The peak number of cases was recorded in one of these months each year except for 1989 when cases peaked in November. The lowest number of cases was recorded in March. Almost identical numbers of diagnoses were confirmed in inpatients as in outpatients at SCHC (80 cases versus 84 cases, respectively). Age of inpatients was signficantly lower than that of outpatients ($P < .001$): the median age of inpatients was 8 weeks; that of outpatients was 28 weeks. Children with Hispanic surnames represented 12% of the cases in 1985 but accounted for 31% to 45% of cases in 1987 to 1990.

The 135 Philadelphia children with pertussis who were brought to SCHC since 1985 resided in 22 different postal zones. Seventy percent of all these patients resided in six postal zones which are major catchment areas for socioeconomically disadvantaged children for whom the institution provides primary care. Seventy-six percent of the Philadelphia cases had families without private health care insurance; this was similar to the institution's 1988 general statistics for those areas of residence. The 29 children from outside Philadelphia resided in 18 postal zones in Pennsylvania and New Jersey; 69% of these families had private health care insurance.

Since January 1985, 96% of inpatients and 90% of outpatients with pertussis have had both culture and DFA tests performed on nasopharyngeal specimens. Sixty-one percent of these patients were positive by both tests. Table 1 shows the tests' relative sensitivities. Culture was more sensitive than DFA testing in confirming pertussis in both inpatients and outpatients, identifying 89% of cases; DFA identified only 72% as positive ($P < .001$). Outpatients were more likely than inpatients to have a negative DFA test ($P = 0.08$). For the 60 children whose diagnoses were confirmed from specimens sent from other hospitals and laboratories, slides for DFA testing alone were sent and were positive in 40. For 17 children who had specimens sent for both tests, culture and DFA had sensitivities of 76% and 71%, respectively, although 5 were positive by culture only and 4 were positive by DFA test only.

Serologic studies. Table 2 summarizes the clinical studies performed and compares clinical, microbiologic, and serologic methods for identifying infected individuals. In an outbreak of pertussis in a residential facility for 66 predominantly immunized children and adults, 64% of residents had confirmed pertussis. Only 24% of the infected individuals had symptoms suggestive of pertussis. Forty-eight percent had positive culture or DFA tests, and 52% were identified only by serologic testing. The study of 18 immunized family members of 4 young children with pertussis identified transmission to 83% of contacts. Only one-third of infected family members had symptoms, 20% had positive culture or DFA tests, and 80% were identified only by serologic

TABLE 1. Sensitivity of culture (C) and DFA testing in 153 children at SCHC with pertussis

	No. of patients	No. with positive results			% Sensitivity	
		C+/DFA+	C+/DFA-	C-/DFA+	C	DFA
Inpatients	77	49	17	11	86	78
Outpatients	76	44	26	6	92	66

All children had both tests performed.

TABLE 2. Identification of infection due to *B. pertussis* in clinical studies using serology

	No. subjects studied	No. with *B. pertussis* infection	Median age of infected	No. infected with symptoms (%)	No. infected with positive culture or DFA (%)	No. infected with positive serology only (%)
Outbreak in residential facility	66	42	21 yr	10 (24)	20 (48)	22 (52)
Family study						
Index cases	4	4	7 mo		4	0
Contacts	18	15	17 yr	5 (33)	3 (20)	12 (80)
Vaccine cohort						
Urban	109	12	16 mo	0 (0)	ND	12
Suburban	223	3	16 mo	0 (0)	ND	3

ND, No data.

testing. In the 2-month study of immunized children beginning at 15 months of age, 11% of 104 urban children and 1.3% of 223 suburban children had spontaneous rises in pertussis antibodies diagnostic of *Bordetella* infection. None had respiratory symptoms with cough of any duration or known exposure to anyone with pertussis. However, 9 of 12 infected urban children lived one to six blocks from a household which had a case of pertussis diagnosed within 1 month of the time of antibody rise.

The results of pertussis antibody tests performed in the three clinical studies are shown in Table 3. All four unimmunized or partially immunized young children who were the index cases of the family study had fourfold or greater rises in antitoxin, PT-G, and FHA-G antibodies (only one child had any of these antibodies measurable at the time of diagnosis). Two had measurable levels of FHA-A some time during the year of follow-up. Only PT antibody levels exceeded high cutoff values relative to the immunized control population at any time during follow-up. Agglutinating antibody was already present in all four children at the time of diagnosis; it rose further in only one child, and did not reach high levels in any child.

More than 85% of immunized uninfected individuals in the family, residential, and toddler serosurvey studies had measurable antibody by agglutinin, antitoxin, PT-G, and FHA-G test. Six percent had measurable antibody by FHA-A test. No single antibody test identified most immunized individuals who had microbiologic or serologic evidence of infection. The FHA-A and PT-G tests were the most effective, with 65% showing a significant change, high value or both for FHA-A and 51% for PT-G. Considering both tests, 92% of the individuals who met serologic criteria for *B. pertussis* infection and 86% who had serologic or microbiologic confirmation had a significant change or high value in at least one test.

Antibody responses of symptomatic immunized individuals were significantly different from those with subclinical infection. Those with symptomatic infection were more likely to have diagnostic PT-G values (93%) and antitoxin (53%) than were asymptomatically infected individuals (40% and 33%, respectively); *P* values were $< .001$ and .15, respectively. Immunized individuals with subclinical infection were somewhat more likely to have diagnostic elevations of FHA-A (68%) and FHA-G (39%) than were symptomatically infected study subjects (53% and 13%, respectively); *P* values = 0.27 and 0.06, respectively.

Immunization status and timing of serum sampling affected the positivity of antibody tests. Significant changes in titer were more likely to be shown between acute and convalescent serum of index cases and in the prospective serosurvey than in case contact studies. For example, significant changes in agglutinin antibodies were found in 80% of immunized infected toddlers in the prospective study, but only 28% of infected contacts (most immunized) in the outbreak and family studies. Similarly, significant changes occurred in FHA-G antibody values in 73% of infected toddlers in the prospective study, but in only 12% of infected contacts in the outbreak and family studies. In the family study, all 15

TABLE 3. Serologic test results in study subjects with *B. pertussis* infection

Clinical category/study	No. of Subjects	Pertussis antibody														
		Agglutinin			Antitoxin			PT-G			FHA-G			FHA-A		
		Δ[b]	[↑][c]	[b] or [c] (%)	Δ[b]	[↑][c]	[b] or [c] (%)	Δ[b]	[↑][c]	[b] or [c] (%)	Δ[b]	[↑][c]	[b] or [c] (%)	Δ[b]	[↑][c]	[b] or [c] (%)
Unimmunized index case	4	1	0	1 (25)	4	2	4(100)	4	4	4(100)	4	0	4(100)	2	0	2(50)
Immunized symptomatic																
Outbreak subject	10	5	4	6	2	5	5	7	6	9	0	0	0	0	5	5
Family contact	5	3	2	3	3	1	3	4	5	5	2	2	2	0	3	3
Total	15			9 (60)			8 (53)			14 (93)			2 (13)			8(53)
Immunized subclinical																
Outbreak subject	32	6	3	7	6	7	12	6	6	10	3	2	5	5	21	21
Family contact	10	2	2	2	3	1	3	6	4	6	2	4	5	2	6	5
Vaccine cohort	15	12	2	12	4	1	4	7	0	7	11	5	12	11	0	12
Total	57			21(37)			19(33)			23(40)			22(39)			39(68)

[a] *B. pertussis* infection is defined as (i) a positive DFA or culture or (ii) two different serologic tests with either a fourfold change in value or one value that exceeds 3 standard deviations above the geometric mean value of an immunized control population.

[b] Number of subjects with a fourfold change in test value.

[c] Number of subjects with a test value that exceeds 3 standard deviations above the geometric mean value of an immunized control population.

infected contacts had at least one diagnostically elevated antibody at the time of diagnosis of the index case. Forty-five percent of subsequent significant changes in antibody titers in these infected contacts were decreases rather than increases. Diagnostically high values of agglutinins or FHA-G were achieved in only 17% of infected individuals, considering all clinical categories and studies. Diagnostically elevated values of PT-G and FHA-A were achieved in 33% and 46% of infected individuals, respectively.

DISCUSSION

Pertussis is endemic in North Philadelphia with a trend of increasing numbers of cases confirmed annually since 1979 and the peak incidence in 1990. Characteristic 3- to 5-year cycles of disease have not been seen (8, 11, 23). The model used to predict the frequency of pertussis cycles considers the balance between addition to the population of susceptible individuals by the birth cohort and removal by natural disease or immunization. Epidemics occur when a critical pool of susceptible individuals accrues, usually every 3 to 4 years in highly vaccinated populations. Increased birth rate or decreased immunization rate shortens cycles. Cases of pertussis in North Philadelphia predominantly occur in a socioeconomically disadvantaged population with poor vaccine update. The trend of increased disease in Hispanic infants is due in part to a population change; it also probably reflects findings of a recent survey that fewer than 30% of 24-month-old Latino children in North Philadelphia are appropriately immunized (R. K. Ross, personal communication). The higher socioeconomic status of patients referred from outside Philadelphia demonstrates that pertussis is not limited to urban or poor children. The summer peak of pertussis which occurs annually in the SCHC experience is not understood.

The young age of infants with pertussis (median age of 8 weeks for hospitalized patients and 28 weeks for outpatients) is an exaggeration of recent national trends and is strikingly different from the prevaccine era when disease peaked in toddler, preschool, and school-age children, with less than 10% of cases occurring in infants under 1 year of age (1, 2, 4, 5). The success of the vaccine strategy of the last 40 years in reducing pertussis cases by more than 97% in the targeted age group conceivably is responsible for the skewed ages of maximum attack rate. Young infants, who are not yet candidates for complete immunization, and adolescents and young adults who may have waning or persistent incomplete immunity following vaccine, are currently the age groups of greatest risk (4, 5, 23). Mounting evidence indicates, however, that very young infants are overrepresented (1, 4, 18). Possible explanations include a relative decrease in the transplacental protection provided by vaccine-immune mothers and an increase in the number of susceptible mothers. There is also, unquestionably, an artifact of case ascertainment, with younger children more likely to have morbid disease for which a diagnosis is sought. The young age of outpatients confirmed to have pertussis in Philadelphia, the almost identical number of diagnoses made in outpatients as inpatients, the relative absence of adolescents, and the large number of older family members with pertussoid symptoms identified around index cases suggest that only a small percentage of actual pertussis cases are brought to medical attention. In fact, comparing active and passive surveillance methods for identifying cases brought to medical attention suggests that passive systems capture only 11% to 17% of such cases (5, 11).

At SCHC, culture is consistently superior to DFA testing for diagnosing acute illness due to *B. pertussis*. Isolation is maximized by education regarding specimen collection, use of 1% casamino acid transport medium, and rapid inoculation onto selective enriched agar. False positive reading of DFA tests is minimized by the experience and constancy of laboratory personnel performing the test and strict adherence to both morphologic and fluorescent criteria for positive interpretation. Sixty-one percent of positive specimens are identified by both tests; however, culture is significantly more sensitive ($P < .001$), identifying 89% of infected individuals. A small number of cases are identified by DFA only, especially when antibiotics are given or specimen handling is not optimal. Timing, degree of illness, and prior immunization status all affect the likelihood of confirming cases and, probably, the comparative sensitivity of each test. SCHC patients were predominantly unimmunized, ill, and had not received antibiotics. Outpatients were more likely to have positive culture but negative DFA test, suggesting a lower infective state compared to children requiring hospitalization.

Development of serologic assays has greatly improved the identification of cases of pertussis negative by culture and DFA test. No single antibody test is a sensitive indicator of disease and able to discriminate between response to vaccine and infection (10, 12, 17, 22, 24). Immunization with whole-cell vaccine induces (i) agglutinating antibodies and ELISA FHA-G antibodies which are variable but moderately high and persistent, (ii) relatively lower levels of antibodies to PT as measured by ELISA PT-G or toxin neutralization, and (iii) little if any IgA response as measured by ELISA PT-A or FHA-A antibody. Pertussis is associated with a brisk response to PT (PT-G and antitoxin) and production of IgA antibody (PT-A and FHA-A) (19, 20, 22, 24, 26). Antibodies of IgM class are variably produced and technically less reliably measured (9, 10); ELISA for PT-G is more sensitive and technically more easily performed than is toxin neutralization (10, 26); ELISA for FHA-A is more sensitive than is PT-A and more reliably measured (22). Therefore, in our experience the most useful serologic tests for diagnosis are ELISA for PT-G and FHA-A: approximately 90% of infected individuals in these studies had diagnostically positive results when one of these assays was positive.

Age, prior immunization, and degree of symptomatology affect antibody response following infection. ELISA FHA-A, a sensitive test for infection in most and discriminatory between infection and vaccination, is not a consistent response of young children and previously unimmunized individuals (21). Only one-half of SCHC incompletely immunized infants with pertussis developed FHA-A antibodies. In our experience and the preponderance of that of others, the degree of symptomatic infection in immunized and unimmunized individuals correlates directly to the degree of rise of antibodies to PT (10, 26).

A single-serum sampling approach to diagnosis requires multiple assays to identify all those infected and comparison to an age-matched, immunization-matched control population to establish diagnostically high values for antibody tests. Timing of serum sampling is important. Agglutinating antibody and FHA-G antibody responses are rapid in immunized and unimmunized individuals. All four unimmunized or partially immunized young children with pertussis had one or more of these antibodies present at the time of diagnosis. In the family study, all infected contacts had a diagnostically high antibody value at the time of diagnosis of the index case; 45% of subsequent significant antibody changes were decreases. A paired-serum sampling approach to diagnosis permits significant changes in titer to be detected when individual values frequently do not exceed high cutoff levels. Defining a significant serologic change is problematic. Setting criteria for infection must attempt to include truly infected and exclude uninfected individuals, a difficult task when neither symptoms, microbiologic tests, nor serologic assays is completely reliable (19).

Serologic studies have added new insights into the concepts of infection transmission and disease protection. Transmission of *B. pertussis* in families, residential facilities, and the community is extensive and is greatly underestimated by the marker of symptomatic disease and by microbiologic testing of clinically suspected cases, especially in immunized individuals. Infected individuals (the majority of whom were immunized) in the outbreak and family studies respectively only had symptoms 24% and 33% of the time; they had positive microbiologic confirmation 48% and 20% of the time. These findings agree with other investigations in similar settings (16, 22).

The importance of subclinical infection in spreading disease is unknown. Adults are known to be important contagious sources of *B. pertussis* (18). Analysis of serologic data from the family study showed that young children were the victims rather than vectors of infection, transmission through the family having occurred before the presentation of the "index" case. The prospective serosurvey of 15-month-old immunized toddlers suggests that infection in the community is common, depending on the level of disease activity. Silent infection with *B. pertussis* was demonstrated in 11% of urban children and 1.3% of suburban children over approximately 2 months' time. In Sweden, where disease is endemic, Granström noted increasing antibody titers with age without history of recurrent disease (9). Vaccination and probably also natural disease confer disease immunity more than they provide protection from infection (6, 9, 23). The association of symptomatic disease with antibody response to pertussis toxin suggests that Pittman's concept of pertussis as a toxin-mediated disease is still valid in light of current knowledge (20). The association of subclinical infection with production of IgA antibody to FHA in previously immunized individuals suggests that such a response may abort infection. The spectrum of *B. pertussis* infection and the role of the bacterium and host in determining colonization and disease are important concepts to consider in developing strategies to control pertussis; scientific understanding of these concepts is still evolving.

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DISCUSSION

DR. THOMAS: Thank you very much for that talk. The result that I found striking is the 11 percent of young children having a serologic rise over a two month period. That would suggest to me that if you followed all children over a year that something round about 50 percent should have suffered asymptomatic infection which I find hard to conceive. Do you have any comments?

DR. LONG: This again is an area of North Philadelphia where we know we have endemic pertussis but I think that is the implication.

DR. THOMAS: Do you have any information, say, about places like Jamaica or somewhere that might represent a similar sociological milieu to the sort of cases that I think you are describing in Hispanic people living in poor environments.

DR. LONG: I do not know that.

DR. HEWLETT: That was a very nice presentation. Obviously, you are sensitive to and interested in the issue of pertussis in adults as well as in children. I wondered in the cases that present to your hospital and are hospitalized or the ones that are seen in outpatients, do you have any data or any feeling for the source of acquisition of their infection in the household or wherever they acquired it and is there a difference between the inner city children and the ones that live in a suburban area?

DR. LONG: From our family studies we have learned that although you thought the young infant was the source, the young infant is almost always the victim in a family. In fact, all of the contacts who were proved to be infected in the family studies had at least one antibody titer. At the time of diagnosis of the child it was greater than three standard deviations among our control population, and more than half of their serologic changes that were significant were decreases so that I think that the family obviously is important. I think it is not the only source. We do find in most children who are admitted with pertussis that there is a family member who has a coughing disease and we frequently prove that it is pertussis. It is rarely thought of as pertussis until the child is recognized.

In the suburbs, it is interesting, I do not know if I have enough or have thought about it enough or looked at it carefully enough to have that opinion, I have the sneaking suspicion it is a little different. We see very frequently that the mother gets the disease after the child but I do not know where the child gets it from. The child is more likely to go to a day care in the community and be exposed to lots and lots of children and this may be a source of infection. I am not sure that that is a careful enough answer but I will look into it.

DR. HEWLETT: When you say family members for the inner city, are you talking about siblings or teenage siblings or parents, grandparents?

DR. LONG: Yes, grandparents, parents usually, those who live within the same four walls frequently so it is not confined by genetics or age.

DR. WIRSING VON KÖNIG: Thank you very much. Finally, we would ask Dr. Manclark to give us the concluding remarks of this symposium.

DR. MANCLARK: The hour is late so I will be very brief. We have had three full days and I would like to take a few moments to thank all of you for your participation and your contributions and wish you a safe journey home. Thank you very much. My best regards to all of you. This symposium is closed.

Poster Session Contributions

Poster Session Contributions

Titles and authors of posters presented during the Symposium are listed. A limited number of complimentary reprints of the published abstracts are available from:

Dr. Charles R. Manclark
Center for Biologics Evaluation and Research
8800 Rockville Pike
Bethesda, Maryland 20892 USA

Copies of the abstracts have been identified with accession number PB90-237041 and may be purchased from the National Technical Information Service.

Posters

Structure-Function Analysis of the S1 Subunit of Pertussis Toxin Using Monoclonal Antibodies — H. BOUX, M. MAKINEN, S. COCKLE, P. CHONG, L. GUCCIARDI, S. LOOSMORE, AND M. KLEIN

A Common Structure of the Active Site of ADP-Ribosylating Toxins — MARIO DOMENIGHINI, CESARE MONTECUCCO, AND RINO RAPPUOLI

The Enzymatically Active A Subunit Is Required for the Leukocytosis-Promoting and Histamine-Sensitizing Activities of Pertussis Toxin — JUAN L. ARCINIEGA, W. NEAL BURNETTE, TIMOTHY D. BARTLEY, VERNON L. MAR, DAVID W. WHITELEY, CHARLES R. MANCLARK, AND DRUSILLA L. BURNS

Localization of the Region of the S1 Subunit Is Required for Efficient Binding of Transducin — G. CORTINA AND J. T. BARBIERI

Protective Immunogenicity of Synthetic Peptides Selected from the Amino Acid Sequence of Pertussis Toxin Subunit S1 — P. ASKELÖF, K. RODMALM, G. WRANGSELL, U. LARSSON, S. B. SVENSON, J. L. COWELL, A. UNDÉN, T. BARTFAI

Structural Studies of Pertussis Toxin — MARY A. TURNER, STELLA FAWCETT, LINDSAY SAWYER, AND SIMON VAN HEYNINGEN

Preliminary X-Ray Crystallographic Analysis of Pertussis Toxin — MALINI RAGHAVAN, JOHN W. GOTTO, JANE V. SCOTT, AND CLARENCE E. SCHUTT

The B Oligomer of Pertussis Toxin: Structure-Function Analysis and the Delineation of Neutralizing Epitopes Using a Panel of Monoclonal Antibodies — H. BOUX, M. MAKINEN, P. CHONG, S. COCKLE, G. ZOBRIST, G. JACKSON, S. LOOSMORE, AND M. KLEIN

Western Blot Analysis Can Fail to Detect the Predominant Neutralizing Epitope in Pertussis Toxin Modified by Site-Directed Mutagenesis — HARVEY R. KASLOW, JOHN D. SCHLOTTERBECK, VERNON L. MAR, AND W. NEAL BURNETTE

Antibody Interactions with Pertussis Toxin — Harvey R. Kaslow, John D. Schlotterbeck, and John Gotto

Mapping of Neutralization Epitopes on the S2 and S3 Subunits of Pertussis Toxin Using Synthetic Peptides — P. Chong, M. Sydor, E. Wu, G. Zobrist, and M. Klein

Binding Properties of Pertussis Toxin — Maarten H. Witvliet, Hidde L. Ploegh, Peter Hoogerhout, and Jan T. Poolman

Studies on the Entry of Pertussis Toxin into Chinese Hamster Ovary Cells — Sally Z. Hausman, Charles R. Manclark, and Drusilla L. Burns

Pertussis Toxin Interacts with Immunologically Relevant Molecules on Human Lymphocytes — Ruud J. Klapmuts, Dominique J. M. Borsboom, Emmanuel J. H. J. Wiertz, and Jan T. Poolman

The Carbohydrate-Recognition Domain Mediating Binding of Pertussis Toxin to Human Cilia and Macrophages Resides in Subunit 2 — K. Saukkonen, N. Burnette, V. Mar, and E. Tuomanen

Mouse-Protective Activities of Monoclonal Antibodies Against Pertussis Toxin — Hiroko Sato and Yuji Sato

Modification of the Immunological Properties of Pertussis Toxin by Chemical Inactivation — Luciano Nencioni, Massimo Bugnoli, Daniele Nucci, Samuele Peppoloni, Ilio Marsili, Maria Teresa de Magistris, and Rino Rappuoli

Chemical Detoxification Studies of Pertussis Toxin — G. Jackson, P. Chong, L. Tan, R. Fahim, S. Cockle, H. Boux, and M. Klein

Alterations in Hepatic Drug Metabolism Following Vaccine Administration to Mice — Sherry Ansher and William Habig

Effect of Pertussis Vaccine or Pertussigen Treatment on the Development of Type 1, Insulin-Dependent Diabetes Mellitus in Animal Models — Shih-Wen Huang, Roberto Luchetta, and Ammon B. Peck

Isolation and Characterization of Calmodulin-Like Protein from *Bordetella pertussis* — M. Nagai, M. Endoh, and Y. Nakase

Tracheal Cytotoxin Displaces Serotonin Binding to Respiratory Epithelial Cells — J. L. Collier and W. E. Goldman

Molecular Structural Requirements for Tracheal Cytotoxin Function — K. M. Erwin, J. L. Collier, G. R. Marshall, and W. E. Goldman

A Comparative Study of Dermonecrotic Toxin Activities of Serotype D *Pasteurella multocida* and the Genus *Bordetella* — Kimberly E. Walker and Alison A. Weiss

Location of Filamentous Hemagglutinin and Pertussis Toxin Antigens of *Bordetella pertussis* by Immunoelectron Microscopy — Hideo Watanabe, Shuichi Kanesaki, Yukio Shukuda, and Hiroshi Mayahara

Adherence of *Bordetella pertussis* on the HeLa Cell Surface — TUEN-YEE WONG AND ADRIANA STEFANCIC

Filamentous Hemagglutinin and the 69-kDa Protein, Pertactin, Promote Adherence of *Bordetella pertussis* to Epithelial Cells and Macrophages — A. BHARGAVA, E. LEININGER, M. ROBERTS, Z. M. LI, S. STIBITZ, I. CHARLES, N. FAIRWEATHER, P. NOVOTNY, C. MANCLARK, AND M. BRENNAN

Construction and Characterization of *Bordetella pertussis* 69-kDa Mutants — M. ROBERTS, J. DE AZAVEDO, E. LEININGER, M. J. BRENNAN, E. L. HEWLETT, A. ROBINSON, N. F. FAIRWEATHER, G. DOUGAN, P. NOVOTNY, AND I. G. CHARLES

Cloning and Expression of an Outer Membrane Protein from *Bordetella pertussis* — P. A. REILLY, L. HOUSE-SUMAVIELLE, T. ECKHARDT, AND J. V. SCOTT

Biochemical and Immunological Studies of a 69-kDa Membrane Protein from *Bordetella pertussis* — JOHN W. GOTTO, PATRICIA REILLY, JAMES J. GIBBONS, JR., JANE V. SCOTT, AND THOMAS ECKHARDT

Molecular Characterization of the Major *Bordetella pertussis* Porin Protein — ZHONG MING LI, JULIE H. HANNAH, MICHAEL J. BRENNAN, SCOTT STIBITZ, AND CHARLES R. MANCLARK

Characterization of a 63-kDa Protein from *Bordetella pertussis* which Is Antigenically Related to a Mycobacterial Heat-Shock Protein — JEANINE L. GOULD-KOSTKA, JUAN L. ARCINIEGA, MARTIN KESSEL, CHARLES R. MANCLARK, AND DRUSILLA L. BURNS

Characterization of Monoclonal Antibodies Against the *Bordetella pertussis* Adhesin, Filamentous Hemagglutinin — ELIZABETH LEININGER, PETER G. PROBST, AND JAMES G. KENIMER

Cell-Mediated Immunity to *Bordetella pertussis* in Mice — JESPER W. PETERSEN, PER H. IBSEN, CARINE CAPIAU, AND IVER HERON

Cell-Mediated Immunity to Pertussis Toxin Subunit S4 After Vaccination of Adult Human Volunteers with the Japanese Acellular Pertussis Vaccine JNIH-3 — JESPER W. PETERSEN, PER H. IBSEN, ARNE HOLM, CARINE CAPIAU, AND IVER HERON

Cell-Mediated Immune Responses to Acellular Pertussis Vaccine — FREDERICK R. VOGEL, THERESA GIAMPAGLIA, THOMAS ECKHARDT, AND JANE V. SCOTT

Immune Response to Pertussis Toxin Subunit S1 Synthesized by *Bacillus subtilis* — R.-M. ÖLANDER, A. MUOTIALA, M. KARVONEN, J. HIMANEN, AND K. RUNEBERG-NYMAN

Multiple T- and B-Cell Epitopes in the S1 Subunit of the Pertussis Toxin Molecule — JORGE R. OKSENBERG, AMRIT K. JUDD, AND LAWRENCE STEINMAN

Human Serological Response to Adenylate Cyclase Toxin of *Bordetella pertussis* — JUAN L. ARCINIEGA, ERIK L. HEWLETT, MARY C. GRAY, ADAMADIA DEFOREST, STEVEN G. F. WASSILAK, IDA M. ONORATO, CHARLES R. MANCLARK, AND DRUSILLA L. BURNS

Characterization of the Antibody Response in Mice Following Immunization with the 69-kDa Protein of *Bordetella pertussis* — S. A. HALPERIN, M. KLEIN, AND G. JACKSON

Frequency Analysis of Memory B Lymphocytes Specific for *Bordetella pertussis* Filamentous Hemagglutinin — DIANA F. AMSBAUGH, CHARLES R. MANCLARK, AND ROBERTA D. SHAHIN

Immunoglobulin Antibodies to Pertussis Toxin, Filamentous Hemagglutinin, and Lipopolysaccharide in Predisease, Postdisease, and Control Blood Samples — MARTA GRANSTRÖM AND GUNNAR GRANSTRÖM

Bordetella pertussis Infection Does Not Suppress Human Serum Immunoglobulin Responses — M. G. THOMAS, P. RICHES, AND H. P. LAMBERT

Detection of Immunoglobulin A Antibodies to Fimbriae, Filamentous Hemagglutinin, Pertussis Toxin, and 69-kDa Outer Membrane Protein in Nasopharyngeal Aspirates from Patients and Contacts with Household Exposure to *Bordetella pertussis* — C. M. MINK, C. B. SWARTZ, M. A. SPRAUER, R. D. SHAHIN, C. R. MANCLARK, AND B. D. MEADE

Development of an Adult Mouse Respiratory Colonization Model to Evaluate the Protective Role of Pertussis Antigens and Vaccines — M. J. QUENTIN-MILLET, B. DANVE, M. MIGNON, L. FOURRICHON, AND F. ARMINJON

Preventive Effects of Filamentous Hemagglutinin-Predominant Pertussis Vaccine Tested in Suckling Mice Infected with *Bordetella pertussis* — TATSUO KATO, TOSHIRO GOSHIMA, AND NATSUKI NAKAJIMA

Protection Against *Bordetella pertussis* Evaluated in an Improved Intranasal Challenge Model — E. SIMOEN, C. CAPIAU, P. HAUSER, AND J. PÊTRE

Passive Transmission of Pertussis Antibodies — TOSHIRO GOSHIMA, NATSUKI NAKAJIMA, HIROFUMI KAKU, YUTAKA ARIMOTO, FUMIE HAYASHI, AND TATSUO KATO

Use of Autologous Promoters for Antigen Expression in *Bordetella pertussis* — SHEENA LOOSMORE, GAVIN ZEALEY, REZA YACOOB, LESLIE BOUX, AND MICHEL KLEIN

The *bvg* Locus of *Bordetella pertussis*: Autoregulation and Control of the Expression of Virulence Factors — ENZO SCARLATO, BEATRICE ARICÒ, ANNA PRUGNOLA, ROY GROSS, AND RINO RAPPUOLI

Strains of *Bordetella pertussis* that Produce Only the B Oligomer of Pertussis Toxin — MARIAGRAZIA PIZZA, MASSIMO BUGNOLI, ROBERTO MANETTI, PAOLO RUGGIERO, LUCIANO NENCIONI, MARIA TERESA DE MAGISTRIS, AND RINO RAPPUOLI

Analysis of Transcription of *Bordetella pertussis* Adenylate Cyclase Gene by Primer Extension and Gene Fusion Studies — MICHAEL J. MORAN AND RICHARD L. FRIEDMAN

Bordetella pertussis Fimbriae: Identification and Characterization of Accessory Genes — ROB WILLEMS, ROB EBBERINK, ANNET PAUL, MARY MATHESON, A. ROBINSON, AND FRITS R. MOOI

Molecular Cloning and Characterization of Outer Membrane Protein Genes of *Bordetella avium* 197 — C. GENTRY-WEEKS, A.-L. HULTSCH, R. CURTISS III, AND J. M. KEITH

Construction of *Bordetella pertussis* Strains Producing a Detoxified Pertussis Toxin — M. A. BLOCH AND D. LAMY

Expression of Native and Mutant Pertussis Toxin in *Bordetella pertussis* from a P_{tac}-*ptx* Promoter Fusion — R. A. DEICH, S. BAKER, K. MOUNTZOUROS, AND B. A. GREEN

Development of a Recombinant Whooping Cough Vaccine — SHEENA LOOSMORE, GAVIN ZEALEY, STEPHEN COCKLE, HEATHER BOUX, RAAFAT FAHIM, AND MICHEL KLEIN

Perspectives on the Epidemiology of Pertussis in the United States, 1980-88 — K. M. FARIZO, S. L. COCHI, E. R. ZELL, P. A. PATRIARCA, S. WASSILAK, AND E. W. BRINK

Evaluation of Susceptibility to *Bordetella pertussis* Infection Using Immunoglobulin Class-Specific Enzyme-Linked Immunosorbent Assay, Chinese Hamster Ovary Cell Neutralization, and Agglutinogen Antibody Assays — P. A. PATRIARCA, R. J. BIELLIK, D. G. BURSTYN, G. SANDEN, E. W. BRINK, A. DEFOREST, AND C. R. MANCLARK

Seroepidemiology of Pertussis Infection in an Urban Childhood Population in Cameroon — T. STROFFOLINI, A. GIAMMANCO, A. CHIARINI, S. TAORMINA, A. SARZANA, G. MAZZA, M. MAGGIO, M. CHIARAMONTE, T. NGATCHU, AND D. LANTUM

Cumulative Incidence of Pertussis in Nonvaccinated 10-Year-Old Children in Sweden, a Nonvaccination Country Since 1979 — J. ISACSON, I. KRANTZ, J. TARANGER, AND B. TROLLFORS

Pertussis Hospitalizations and Mortality, United States, 1985-87: Evaluation of the Completeness of National Reporting — R. W. SUTTER, S. L. COCHI, K. M. FARIZO, AND E. R. ZELL

Evaluation of Clinical Case Definitions for Pertussis — P. STREBEL, K. FARIZO, I. ONORATO, P. PATRIARCA, B. ZELL, AND S. COCHI

In Vitro Susceptibility of *Bordetella pertussis* to New Oral Antibiotics (Macrolides, Cephalosporins, and Quinolones) — J. E. HOPPE, A. EICHHORN, J. MÜLLER, AND C. G. SIMON

Diagnosis of *Bordetella pertussis* Infection Using Monoclonal Antibodies in Immunofluorescence: A Comparison with Culture and Immunofluorescence Using Polyclonal Antibodies — K. BROMBERG, C. GULLANS, G. TANNIS, Z. M. LI, M. BRENNAN, AND C. MANCLARK

New, Practical Approach to Detect Filamentous Hemagglutinin Antibody — HIROFUMI KAKU, TOSHIRO GOSHIMA, NATSUKI NAKAJIMA, AND TATSUO KATO

Enzyme-Linked Immunosorbent Assays for Antibodies to *Bordetella pertussis* by Using Antigen-Coated Polystyrene Balls as a Solid Phase — YUJI SATO, HIROKO SATO, HIROHIDE KODAMA, MASAKO UCHIMURA, NORINAGA MIWA, TAKASHI KOBAYASHI, EIJI YAMAMOTO, AND ISAO FUJITA

Development of *Bordetella pertussis* Antibodies in Children with Whooping Cough — P. NOVOTNY, M. E. MACAULAY, C. A. HART, AND F. SKVARIL

A Standardized Enzyme-Linked Immunosorbent Assay Procedure for Quantitation of Immunoglobulin G Antibodies to Pertussis Toxin and Filamentous Hemagglutinin — B. D. MEADE, H. O. HALLANDER, T. A. ROMANI, L. MARDIN, C. B. SWARTZ, M. LJUNGMAN, B. REIZENSTEIN, R. MÖLLBY, W. C. BLACKWELDER, AND C. R. MANCLARK

Enzymatic Gene Amplification for the Specific Detection of *Bordetella pertussis* — JORGE R. OKSENBERG AND LAWRENCE STEINMAN

Specific Identification of *Bordetella pertussis* in Clinical Specimens Using the Polymerase Chain Reaction — A. BOLLEN, S. HOUARD, C. HACKEL, P. JACOBS, A. HERZOG, A. DELEM, J. PÊTRE, AND F. AMBROSCH

Knowledge, Attitudes, and Practices Affecting DTP Vaccination Among Infants and Children in Maricopa County, Arizona, 1988 — M. A. SPRAUER, S. L. COCHI, P. A. PATRIARCA, E. R. ZELL, R. W. SUTTER, J. R. MULLEN, AND S. J. ENGLENDER

Efficacy of Whole-Cell Pertussis Vaccine: Estimates from the Pertussis Multicenter Surveillance Project — IDA M. ONORATO, ELIZABETH R. ZELL, STEVEN G. WASSILAK, EDWARD W. BRINK, AND ROGER H. BERNIER

Epidemiological Evidence of Suboptimal Pertussis Vaccine — PETER CAMPBELL, PETER MASTERS, DAVID MCKAY, AND ZLATA WALLNER

Differences in Antibody Response to Whole-Cell Pertussis Vaccines — KATHRYN M. EDWARDS, NEAL A. HALSEY, MICHAEL D. DECKER, AND BARRY AUERBACH

Antigenic Composition of the Danish Whole-Cell Pertussis Vaccine — P. M. IBSEN, J. W. PETERSEN, AND I. HERON

Potentiation of Tetanus Toxoid Potency by Pertussis Vaccine — K. REDHEAD, T. HILL, J. WATKINS, AND R. E. GAINES DAS

Severe DTP-Associated Reactions — DEAN A. BLUMBERG, CHRISANNA M. MINK, KAREN LEWIS, PATRICIA CHATFIELD, LINDA P. SMITH, CHARLES LEACH, PETER D. CHRISTENSON, W. DONALD SHIELDS, SEYMOUR R. LEVIN, MARY BETH J. STEINFELD, LARRY J. BARAFF, NANCY SCHONFELD, AND JAMES D. CHERRY

Confounding in Studies of Adverse Reactions to Vaccines — PAUL E. M. FINE AND ROBERT CHEN

The National Childhood Encephalopathy Study: A 10-Year Followup — N. MADGE, D. MILLER, E. ROSS, AND J. WADSWORTH

Acute Neurological Illness and DTP: Report of a Case-Control Study in Washington and Oregon — JAMES L. GALE, PURU B. THAPA, JANET K. BOBO, STEVEN G. F. WASSILAK, PAUL M. MENDELMAN, AND HYORDIS M. FOY

Pertussis Toxoid Inactivated with Tetranitromethane: Safety and Immunogenicity in Adults, Children, and Infants — G. R. SIBER, L. HERZOG, C. D. MARCHANT, N. COHEN, L. W. WINBERRY, AND C. W. TODD

Lack of Antibody Response to 69-kDa Outer Membrane Protein, Fimbriae, and Lipooligosaccharide of *Bordetella pertussis* in Children Enrolled in the Efficacy Trial of Pertussis Vaccine in Sweden — T. A. ROMANI, C. B. SWARTZ, H. HALLANDER, P. OLIN, J. STORSAETER, C. R. MANCLARK, AND B. D. MEADE

Biological and Protective Properties of the 69-kDa Outer Membrane Protein of *Bordetella pertussis*. A Novel Formulation for Acellular Pertussis Vaccine — P. NOVOTNY, A. P. CHUBB, K. COWNLEY, AND I. G. CHARLES

Pertussis and Pertussis Immunization in Japan — MIKIO KIMURA AND HARUMI KUNO-SAKAI

Japanese Clinical Trials with Takeda Acellular Pertussis Vaccine — HARUMI KUNO-SAKAI, MIKIO KIMURA, SHIN ISOMURA, HITOSHI KAMIYA, RITSUE NII, TATSUO KATO, AND ISAO FUJITA

Development and Characterization of an Acellular DTP Vaccine — CHUN N. SHIH, FRED F. TUNG, GEORGE H. BURGOYNE, ROBERT B. BELSHE, EDWIN L. ANDERSON, AND JOHN R. MITCHELL

A Novel, Rapid Nonaffinity Method to Simultaneously Purify *Bordetella pertussis* Toxin and Filamentous Hemagglutinin — LARRY U. L. TAN

Guinea Pig Immunogenicity Study of a Tetravalent Pertussis Vaccine — R. FAHIM, G. JACKSON, J. VOSE, G. ZOBRIST, L. TAN, D. MCEACHRAN, J. BEVILACQUA, A. HERBERT, L. BOUX, H. BOUX, AND M. KLEIN

Development of Acellular Component Pertussis Vaccines — J. PÊTRE, C. CAPIAU, AND H. BOGAERTS

Comparison of Antigenicity of Four Different Acellular DTP Vaccines with Single Radial Hemolysis Assay — H. IWAI, Y. MURASE, R. GOTO, T. GONDA, T. AOYAMA, AND T. IWATA

Pertussis in the Adult — T. AOYAMA, R. GOTO, H. IWAI, Y. MURASE, AND T. IWATA

Multiple Approaches to Estimating the Incidence of Pertussis in a Part of Northrhine-Westphalia, Federal Republic of Germany — M. SCHMITZ, S. G. WASSILAK, AND C. H. WIRSING VON KÖNIG

Dose Response to Acellular Pertussis Vaccine and Comparison with Whole-Cell Pertussis Vaccine at 15 to 24 Months and 4 to 6 Years of Age — BARRY S. AUERBACH, MODENA E. WILSON, ALAN M. LAKE, ADAMADIA DEFOREST, AND NEAL A. HALSEY

Booster Response to Acellular Vaccines in Children Primed with Acellular or Whole-Cell Vaccine — KATHRYN M. EDWARDS, MICHAEL D. DECKER, AND ROBERTA BRADLEY

Safety and Immunogenicity of a Tetranitromethane Pertussis Toxoid in 14- to 18-Month-Old Swiss Children — S. J. CRYZ, JR., G. R. SIBER, N. COHEN, L. WINBERRY, C. TODD, R. BERGER, AND M. JUST

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Allietta, Margaretta, **13**
Allison, Neil, **202**
Ambrosch, F., **352**
Amsbaugh, Diana, **196, 350**
Anderson, Edwin, **353**
Angus, William C., **57**
Ansher, Sherry, **348**
Aoyama, Tatsuo, 320, 321, **353**
Arciniega, Juan L., 156, 165, **347, 349**
Aricò, Beatrice, **350**
Arimoto, Yutaka, **350**
Arminjon, François, **350**
Ashworth, L. A. Edward, **303,** 309, 310, **330**
Askelöf, P., **347**
Au Jensen, Maria, **136**
Auerbach, Barry S., **352, 353**
Avigan, Joel, **57**

Baker, S., **351**
Ballantine, S., **275**
Baraff, Larry, **352**
Barbieri, Joseph T., **347**
Barlow, Ann K., **148**
Barnard, Amanda, **166**
Barry, Eileen M., **260,** 266
Bartfai, T., **347**
Bartley, Timothy D., **347**
Bartoloni, Antonella, **37**
Beesley, Katrina, **136**
Bellalou, Jacques, **233**
Belshe, Robert B., **353**
Bemis, David A., 36, 114
Berg, Douglas E., **243**
Berger, R., **353**
Bernier, Roger H., 301, 302, **311,** 313, 314, **352**
Bevilacqua, J., **353**
Bhargava, Amit, **349**
Biellik, R. J., **351**
Björksten, Bengt, **184**
Blackwelder, William C., **295, 351**
Blennow, Margareta, **184,** 188
Bliziotes, M. Michael, **57**
Bloch, Marie-Aline, **350**
Blumberg, Dean A., **175, 352**

Bobo, Janet K., **352**
Bogaerts, Hughes, **353**
Bollen, Alex, **352**
Borsboom, Dominique J. M., **348**
Bossu, P., **161**
Boux, Heather, **188, 347, 348, 351, 353**
Boux, L., **350, 353**
Bradley, Roberta, **353**
Brennan, Michael J., 28, **89, 100, 136, 141,** 147, **349**
Brink, E. W., **351, 352**
Bromberg, Kenneth, 124, **351**
Bugnoli, Massimo, **37, 348, 350**
Burgoyne, George H., 353
Burnette, Neal W., 40, **347, 348**
Burns, Drusilla L., **3, 30, 53, 347, 348, 349**
Burns, Eugene H., 11, 40, 56, 65, 73, 74
Burstyn, Don G., **351**

Cabezon, Teresa, **41**
Cameron, Jack, 302, 314
Campbell, Peter, **352**
Capiau, Carine, **75,** 85, 86, **349, 350, 353**
Carr, Steven A., **75**
Chandler, Donna K. F., 201
Charles, Ian G., **136, 275, 349, 352**
Chatfield, Patricia C., **175, 352**
Chen, Joseph K., 302
Chen, Robert, **352**
Cherry, James D., **175,** 301, 310, **352**
Chiaramonte, M., **351**
Chiarini, Alfredo, **351**
Chong, Pele, **347, 348**
Christenson, Peter D., **175, 352**
Chubb, A. P., **352**
Cieplak, Witold Jr., **281**
Cochi, Stephen L., **351, 352**
Cockle, Stephen A., **347, 348, 351**
Cohen, Nadine D., **352, 353**
Collier, Jayne, **5, 348**
Comberbach, Martin, **75**
Conrath, Karel, **75**
Cookson, Brad, **5,** 19, **243,** 250
Cortina, Galen, **347**
Covacci, Antonello, **37**

Cowell, James L., 52, 105, 147, 156, 347
Cownley, K., 352
Cryz, Stan J., 188, 353
Curtiss, Roy, 189, 350

Danve, B., 350
Das, R. E. Gaines, 352
De Azavedo, J., 349
De Magistris, M. Teresa, 37, 159, 161, 348, 350
Decker, Michael D., 352, 353
Deforest, Adamadia, 335, 349, 351, 353
Deich, Robert A., 351
Delem, A., 352
Desmons, Pierre, 75
Di Tommaso, A., 161
Domenighini, Mario, 347
Dougan, G., 136, 275, 349

Ebberink, Rob, 350
Eckhardt, Thomas, 286, 349
Edwards, Kathryn M., 309, 352, 353
Ehrmann, Ingrid E., 13, 28, 260
Eichhorn, A., 351
Endoh, Masahiko, 30, 36
Endoh, Mayumi, 196, 348
Englender, S. J., 352
Erwin, Kathryn M., 5, 348
Ewanowich, Carol, 106, 124
Ewell, John B., 141

Fahim, Raafat, 348, 351, 353
Fairweather, N.F., 280, 349
Falkow, Stanley, 91, 217
Farizo, Karen M., 351
Farrington, Conor P., 291
Fawcett, Stella, 347
Fine, Paul E. M., 352
Finger, Horst, 315
Finn, Theresa, 115, 155, 156
Fisher, Margaret C., 335
Fourrichon, L., 350
Foy, Hyordis M., 352
Fremgen, Peter R., 13
Friedman, Richard L., 11, 29, 124, 350
Fujita, Isao, 351, 353
Funnell, S. G. P., 126

Galazka, Artur, 310
Gale, James L., 352
Gentry-Weeks, Claudia R., 350
Giammanco, Anna, 351
Gibbons, James J. Jr., 349
Giampaglia, Theresa, 349
Goldman, William E., 5, 10, 11, 36, 56, 206, 243, 348
Gonda, T., 353
Goodwin, Matina S., 202, 260
Gordon, Valery M., 13
Goshima, Natsuki Toshiro, 350, 351
Goto, Akina, 353
Gotto, John W., 85, 347, 348, 349
Gould-Kostka, Jeanine L., 349
Goyard, Sophie, 233
Granström, Gunnar, 350
Granström, Marta, 184, 350
Gray, Lloyd, 13
Gray, Mary R., 13, 260, 349
Green, B. A., 351
Gross, Roy, 350
Gucciardi, L., 347
Guiso, Nicole, 205, 207, 211
Gullans, C., 351
Gupta, Rajesh K., 19, 36, 40, 85, 86, 124, 301, 302

Habig, William H., 11, 348
Hackel, C., 352
Hallander, H., 291, 351, 352
Halperin, Scott A., 349
Halpern, Jane L., 57
Halsey, Neal A., 352, 353
Hamstra, Hendrik-Jan, 148
Hannah, Julie H., 141, 349
Hanski, Emanuel, 18, 20, 28, 29, 51, 113, 123, 205
Harbert, K. A., 303
Hart, C. A., 351
Hauser, Pierre, 75, 350
Hausman, Sally Z., 53, 348
Hayashi, Fumie, 350
Hemling, Mark E., 75
Herbert, A., 353
Heron, Iver, 136, 349, 352
Herzog, L., 352
Hewlett, Erik L., 11, 13, 18, 19, 36, 114, 211, 260, 342, 343, 349
Hill, T., 352
Himanen, J., 349
Holm, Arne, 349
Hoogerhout, Peter, 348
Hoppe, Jorg E., 351
Houard, S., 352
House-Sumavielle, L., 349

Huang, Shih-Wen, 165, 178, 188, **348**
Hultsch, A.-L., **350**

Ibsen, Per Harding, **349, 352**
Irons, L. I., **126**
Isacson, Jerker S., **351**
Isomua, Shin, **353**
Iwai, Hideto, **353**
Iwata, T., **353**

Jackson, Gail E. D., **347, 348, 349, 353**
Jacobs, P., **352**
Judd, Amrit, **161, 349**
Just, Max M., **353**

Kaku, Hirofumi, **350, 351**
Kamiya, Hitoshi, **353**
Kanesaki, Shuichi, **348**
Karvonen, M., **349**
Kaslow, Harvey R., 206, **347, 348**
Kato, Tatsuo, **350, 351, 353**
Keith, J. M., **350**
Kelly, Sandra M., **189**
Kenimer, James, **100, 106, 349**
Kessel, Martin, **141, 349**
Kimura, Mikio, **353**
Klapmuts, Ruud J., **348**
Klein, Michel, **347, 348, 349, 350, 353**
Knight, Peter A., 232, 250
Kobayashi, Takashi, **351**
Kodama, Hirohide, **351**
Krantz, I., **351**
Kuipers, Betsy, **148**
Kuno-Sakai, Harumi, **353**

Ladant, Daniel, **233**
Lake, Alan M., **353**
Lamb, David, 310
Lambert, Harold P., **330, 350**
Lamy, D., **350**
Lantum, D., **351**
Laoide, Brid M., **233**
Larsson, U., **347**
Leach, Charles, **352**
Lee, Cynthia K., **115**
Lee, Shwu-Maan, 123, 124
Leininger, Elizabeth, 18, 51, **100**, 105, **106**, 206, 211, **349**
Levin, Seymour R., **352**
Lewis, Karen, **352**
Li, Li Jing, **136**
Li, Zhong Ming, **141, 349, 351**
Lindsay, Diane S. J., **179**
Ljungman, M., **351**
Lobet, Yves, **281**, 286
Locht, Camille, **41**, 51, 52, 99, 231, 241, 286
Loggen, Henk, **148**
Long, Sarah S., 314, **335**, 342, 343
Loosmore, Sheena M., **347, 350, 351**
Luchetta, Roberto, **348**

Macaulay, M. E., **351**
Madge, Nicola, **352**
Maggio, M., **351**
Makinen, M., **347**
Makoff, A. J., **275**
Manclark, Charles R., ***v*, 30, 53, 136, 141, 196**, 322, 343, **347, 348, 349, 350, 351, 352**
Manetti, Roberto, **37, 350**
Mar, Vernon L., **347, 348**
Marchant, Colin D., **352**
Mardin, L., **351**
Marshall, G. R., **5, 348**
Marsili, Ilio, **348**
Marzillier, Jutta, **66**
Masters, Peter, **352**
Masure, H. R., 18, 28, 99, 114, **267**
Matheson, Mary, **126, 350**
Mayahara, Hiroshi, **348**
Mazza, G., **351**
McEarchran, D., **353**
McKay, David, **352**
Meade, Bruce D., 188, **289**, 309, 310, **322**, 329, 333, **350, 351, 352**
Mekalanos, John J., **115**
Meller, Ruth, **20**
Mendelman, Paul, **352**
Mignon, M., **350**
Miller, David L., **303, 352**
Miller, Elizabeth, **303, 330**
Miller, Jeff F., **217**, 309, 313
Mills, Kingston H. G., **166**
Mink, ChrisAnna M., **175**, 310, **322, 350, 352**
Mitchell, John R., **353**
Miwa, Norinaga, **351**
Molina, Christine N., **189**
Möllby, R., **351**
Montecucco, Cesare, **347**
Mooi, Frits, **251**, 257, 258, 259, **350**
Moran, Michael J., **350**
Mortensen, Joel E., **335**

Moss, Joel, **57**
Mountzouros, K., **351**
Mullen, J. R., **352**
Müller, J., **351**
Muotiala, A., **349**
Murase, Yuji, **353**
Murtagh, James J. Jr., **57**

Nagai, Masaaki, **30, 348**
Nagel, Jaap, **148**
Nakajima, Natsuki, **350, 351**
Nakase, Yasukiyo, **30, 348**
Nencioni, Luciano, **37, 161, 348, 350**
Newman, Kenneth B., **57**
Ngatchu, T., **351**
Nguyen, Nga Y., **141**
Nii, Ritsue, **353**
Novotny, Pavel, **136, 349, 351, 352**
Nucci, Daniele, **348**
Nuti, S., **161**

Oksenberg, Jorge, **161, 349, 352**
Ölander, R.-M., **349**
Olin, Patrick, **299,** 301, 302, **352**
Olivieri, Roberto, **37**
Onorato, Ida M., **349, 351, 352**
Oxer, M. D., **275**

Parker, Charlotte D., 40, 51, 105, 123, **189,** 194, 195, 200, 286, 320
Parton, Roger, **179**
Patriarca, Peter A., **351, 352**
Paul, Annet, **350**
Pearce, A., **126**
Peck, Ammon, **348**
Peppler, Mark S., 11, 99, **106,** 113, 114, 206
Peppoloni, Samuele, **348**
Permanne, Philippe, **75**
Perugini, Maria, **37**
Petersen, Jesper W., **349, 352**
Pétre, Jean O., **75,** 334, **350, 352, 353**
Pineda, Evelyn, **175**
Pittman, Margaret, 259
Pizza, Mariagrazia, **37, 350**
Plainchamp, Dominique, **75**
Ploegh, Hidde, **348**
Podda, A., **161**
Poolman, Jan T., 10, 11, 29, **148,** 156, 310, **348**
Prebula, Randy J., 165
Price, S. Russ, **57**
Probst, Peter G., **349**
Prugnola, Anna, **350**

Quentin-Millet, Marie-Josée, 86, 147, 258, 310, **350**
Quinnan, Gerald V. Jr., *vi*

Raghavan, Malini, **347**
Rappuoli, Rino, **37,** 40, 86, **161,** 211, 224, 231, 232, 258, 259, **347, 348, 350**
Raupach, Bärbel, **66**
Redhead, Keith, **166, 352**
Reilly, Patricia A., **349**
Reizenstein, B., **351**
Relman, David A., **91,** 99, 124, 242
Relyveld, Edgar H., 187
Riches, P., **350**
Roberts, Angela L., **115**
Roberts, M., **275, 349**
Robinson, Andrew, **126,** 135, **349, 350**
Rocancourt, Murielle, **207**
Rodmalm, K., **347**
Roelants, Piet, **75**
Rogel, Arie, **20**
Romani, Theresa A., **351, 352**
Romanos, Michael, **136**
Ross, Euan M., **352**
Roy, Craig, **217,** 224, 232, 274
Ruggiero, Paolo, **37, 350**
Runeberg-Nyman, Kate T., **161, 349**

Sakamoto, Hiroshi, **233**
Sanchez, A., **161**
Sande, Stephen, **267**
Sanden, Gary N., **351**
Sarzana, A., **351**
Sato, Hiroko, **348, 351**
Sato, Yuji, 40, 188, 310, **348, 351**
Saukkonen, Kirsi, 52, **91, 267, 348**
Sawyer, Lindsay, **347**
Scarlato, Enzo, **350**
Schlotterbeck, John D., **347, 348**
Schmidt, M. Alexander, **66,** 73, 74
Schmitz, M., **353**
Schonfeld, Nancy, **352**
Schutt, Clarence E., **347**
Scott, Jane V., 40, **347, 349**
Seabrook, R. N., **126**
Serventi, Inez M., **57**
Shahin, Roberta D., 51, 113, 135, 165, 174, 183, 188, 194, 195, **196,** 200, 201, 206, 257, **350**

Shields, W. Donald, **352**
Shih, Chun N., **353**
Shukuda, Yukio, **348**
Siber, George R., **352, 353**
Simoen, Etienne, **75, 350**
Simon, C. G., **351**
Sizaret, Phillipe P., 302
Skvaril, F., **351**
Smith, Linda P., **175, 352**
Sprauer, Mary Ann, **350, 352**
Stainer, Dennis, 201
Stefancic, Adriana, **349**
Steinfeld, Mary Beth J., **352**
Steinman, Laurence, **161, 349, 352**
Steven, Alasdair, 135, **141**
Stevens, Linda, **57**
Stibitz, E. Scott, **215, 225,** 231, 232, 258-259, 274, 280, **349**
Storsaeter, J., **291,** 301, **352**
Strebel, Peter M., **351**
Stroffolini, Tommaso, **351**
Strugnell, Richard, **136**
Sutter, Roland, **351, 352**
Svenson, S. B., **347**
Swartz, Christina B., **350, 351, 352**
Sydor, Margaret M., **348**
Szatanik, Marek, **207**
Szulczynski, Miroslav, **66**

Tacken, Angelika, **315**
Tagliabue, A., **161**
Tan, Larry U. L., **353**
Tannis, G., **351**
Taormina, S., **351**
Taranger, John, **351**
Thapa, Puru B., **352**
Thomas, Mark G., 36, 206, 241, 258, **330,** 333, 334, 342, **350**
Todd, C. W., **352, 353**
Tomcsanyi, Tihamer, **243**
Trollfors, Birger, **351**
Tsai, Su-Chen, **57**
Tsuchiya, Mikako, **57, 65**
Tung, Fred F., **353**
Tuomanen, Elaine I., **91,** 99, 105, 274, **348**
Turner, Mary A., **347**

Uchimura, Massako, **351**
Ullmann, Agnes, 18, 52, 135, 211, 224, 231, **233,** 241, 242, 250, 266
Undén, A., **347**
Van Der Heide, Han G. J., **251**
Van Heyningen, Simon, **347**
Vaughan, Martha, **57**
Vogel, Frederick R., **349**
Vose, J., **353**

Wadsworth, J., **352**
Waight, P. A., **303**
Walker, Kimberly E., **348**
Walker, Mary W., **57**
Wallner, Zlata, **352**
Wardlaw, Alastair C., **179,** 232
Wassilak, Steven G., **349, 351, 352, 353**
Watanabe, Hideo, **348**
Watkins, J., **166, 352**
Weiss, Alison A., 114, **202,** 205, 206, 224, **260,** 334, 34
Whiteley, David W., **347**
Wiertz, Emmanuel J.H.J., **348**
Willems, Rob, **251, 350**
Wilson, Modena E., **353**
Winberry, Larry, **352, 353**
Wirsing von König, C. H., **315, 353**
Witvliet, Maarten H., **348**
Wolff, Jan, 19, 28, 124
Wong, Tuen-Yee, **349**
Wrangsell, G., **347**
Wright, Samuel D., **91**
Wu, E., **348**

Yacoob, Reza, **350**
Yamamoto, Eiji, **351**
Yang, Mei-Shin, 225
Yu, Jinan, **189**

Zealey, Gavin, **350, 351**
Zell, B., **351**
Zell, Elizabeth R., **351, 352**
Zobrist, Gloria, 347-348, **353**

Subject Index

Subject Index

adenylate cyclase, antigen, protective 207
adenylate cyclase toxin 13,20
  detection of *B. pertussis* 318
  disease process 202
  domain structure 260
  immune response 176
  non-toxic form 28
  regulation 267
adenylate cyclase-hemolysin 233
  regulation and secretion 243
adherence
  antifimbrial antibodies 133
  fimbriae 126
  genetics, *B. pertussis* 91
  microfilament disrupters 110
  natural host 95
  surface proteins 100
ADP-ribosylating toxins; pertussis toxin 53
ADP-ribosylation
  clustering, Chinese hamster ovary cells 56
  G proteins 57
ADP-ribosyltransferase, S13 down regulation 285
adverse reactions
  acellular pertussis vaccines 311
  Arthus, or delayed type 39
  chemical detoxification 39
  PT-9K/129G 162
  swelling 309
  vaccines 185
  whole-cell and acellular DTP vaccines 306
aerosol challenge
  mice 166
  mouse model 196
  porin model 144
agglutinogens 2 and 3, immune response 330
  T-cell response 166
aluminum, vaccines 187
amino-terminal region, filamentous hemagglutinin 48
anti-Agg 2/3 330
  Merieux and Lederle vaccine 310
  vaccines 304
anti-FHA 324, 330
  Swedish efficacy trial 292
  ELISA, cross-reactivity 294
  vaccines 304
anti-PT 324, 330
  Swedish efficacy trial 292
  vaccines 304
antibodies
  filamentous hemagglutinin (FHA) 48
  IgA 198
  IgA nasal secretions 330
  IgA oral vaccines 195
  IgE 179
  IgE acellular vaccine 184
  IgE clinical trials 310
  IgG 198, 324
  IgG ELISA, pertussis infections 292
  IgG vaccines 304
  IgM 324
  induced, porin 141
  Salmonella typhimurium vaccines 189
  serodiagnosis, pertussis 323
  TCT derivatives 9
  toxin neutralizing 38, 199
antibody response
  mucosal immunity 197
  patients with pertussis infections 338
  whole-cell and acellular vaccines 303
antigenic variation, *B. pertussis* 258
antigens, acellular pertussis vaccines 312
antigens, serodiagnosis, pertussis 323
arachidonic acids
  heat-labile toxin, inhibitory factors 34
Arthus reactions, chemical detoxification 39
assays
  AC enzymatic activity 21
  AC hemolytic activity 22
  AC toxin 22, 234, 261
  adenylate cyclase 234, 244, 261
  adherence 107
  ADP-ribosylation 53
  attachment 117
  bacterial attachment 102
  bactericidal 150
  beta-galactosidase 218
  cell attachment 101
  cellular proliferation, whole-blood 175
  Chinese hamster ovary cell 56, 291
  Chinese hamster ovary, detoxified PT 38
  Chinese hamster ovary, serodiagnosis 325
  cytotoxicity 53
  DNA 217
  heat-labile toxin 30
  hemolytic activity 234
  IgE, detection methods 182

assays (cont'd)
  IL-2/IL-4 167
  in vitro cell adhesion 133
  intracerebral challenge, detoxified PT 39
  invasion 106, 117
  ischemia and dermonecrotizing activities 30
  limiting dilution adoptive transfer 198
  microagglutination, serodiagnosis, pertussis 325
  PCA 180
  proliferation 167
  serologic, pertussis 323, 340
  T cell 167
  TCT production 5
  Vero cell adhesion 128
B lymphocytes, memory response 197
B oligomer, pertussis toxin 40, 46
B. bronchiseptica
  heat-labile protein, molecular weight 36
  repeat sequence motifs 136
B. parapertussis
  anti-FHA IgG, *B. pertussis* 294
  heat-labile protein, molecular weight 36
  isolation 315
  repeat sequence motifs 136
  strains 23054 30
B. pertussis mutants
  adhesion 130
  reversion rates 40
B. pertussis species
  BPRA 46
  Tohama I 5, 46
  Tohama III 6
B. pertussis strains
  101 94
  101-TOX6 94
  10901 141
  1098 94
  134 148
  18323 76, 101, 148, 197, 207, 233
  338 106
  347 141
  509 148
  509 mutants 148
  536 94
  537 94
  adhesins 92
  BP338 13, 203
  BP347 203
  BP348 203, 263
B. pertussis strains (cont'd)
  BP348 pRMB1 20
  BP353 (FHA$^-$) 179
  BP357 (PT$^-$) 179
  BP357 203
  BP536 226
  mutants 203, 226, 233, 254
  Tohama 148
  Tohama BP536 (serotype $2^+,3^-$) 252
  Tohama I 75, 101
  TOX6 148
  W28 148
  W28(69K$^-$) 148
  Wellcome 28 (serotype 1, F2, 3) 126
  Wellcome 28 254
  Wellcome 28 phase 1 166
B. pertussis
  clinical specimens, detection 315
  heat-labile protein, molecular weight 36
  isolation 315
bacterial colonization, adenylate cyclase 207
bacterial isolation, Swedish efficacy trial, rates 292
binding sites
  AC toxin and calcium 18
  adherence 103
  filamentous hemagglutinin (FHA) 47
  macrophage, CR3 94
  NAD, pertussis toxin S1 subunit 44
  pertussis toxin, S3 subunit 66
  T lymphocytes, S1 subunit 164
  TCT 9
biosynthesis, TCT 5
bovine serum albumin, heat-labile toxin, lesions 32
British whole-cell vaccines, clinical trials 303
bvg locus see *vir* locus
C-mode *B. pertussis*, vaccines 154
C-terminal region, 69-kDa protein 76, 276
C-terminal region, pertussis toxin, S1 subunit 42
calcium
  AC toxin 13
  AC toxin induced cAMP generation 24
  CyaA protein 235
calmodulin (CaM), AC toxin 18
calmodulin (CaM), AC toxin, hemolytic activity 25
cAMP, AC toxin 20
cAMP, AC toxin and calcium 13
CAMR acellular vaccine 308
carbohydrate recognition domain, eukaryotic 52
carboxy-terminal region, FHA 48

carboxy-terminal region, G proteins 58
case definitions
  clinical trials 312
  pertussis vaccines 301
  vaccine efficacy 300
  vaccine efficacy, clinical trials 297
cell morphology, microfilament disrupters 107
cellular immune response
  aerosol-infected mice 166
  detoxified pertussis toxin 161
  patients (*B. pertussis*) 175
  S1 subunit 163
cephalexin 320
chemical detoxification, antibody quality 39
Chinese hamster ovary cells 54, 100
Chinese hamster ovary cells, invasion, *B. pertussis* 115
cholera toxin, eukaryotic cells, entry 54
cholesterol, Tohama I and III 105
chromosomal mapping, transposon insertions 270
ciliated cells, adherence 91
ciliated cells, TCT 5, 11
cleavage sites, 69-kDa protein 80
clinical criteria, pertussis vaccine efficacy 295
clinical specimens
  B. pertussis detection 315
  B. pertussis structural components 318
clinical symptoms, vaccine efficacy 297
clinical trials
  antigens and route 302
  double blind trials 302
  IgE response, pertussis toxin 184
  outstanding issues 311
  PT-9K/129G 39, 161
  serodiagnosis 325
  Swedish efficacy trial 291, 295, 299
  United Kingdom 303
colonization, pertussis vaccines 300
Cotton effect, Fim 2 and 3 129
coughing spasms
  vaccine efficacy, clinical criteria 295
culture confirmation, vaccine efficacy 297, 300
cysteine residues, pertussis toxin, S1 subunit 43
cytochalasin B, pertussis and diphtheria toxin 55
cytochalasins, adherence 111
cytotoxicity, pertussis toxin S1 subunit 46
DAP, TCT function 8
diagnosis, pertussis 322
diagnosis, Swedish efficacy trial 291
diagnostic methods, pertussis 335
diphtheria toxin
  eukaryotic cells, entry 54
  NH_4Cl and Chinese hamster ovary cells 55
disease process, *B. pertussis*, isolation 318
dissociation, fimbriae, *B. pertussis* 133
DPT vaccines see vaccines, DTP
E. coli, P.69 protein, expression and purification 275
ELISA
  anti-FHA, IgG 294
  diagnosis, pertussis infections 291
  fimbriae 127
  IgE 182
  pertussis toxin, detoxified 38
  S3 subunit, peptides 67
  serodiagnosis, pertussis 322
endocytosis, cytochalasin B 55
endotoxin, heat-labile toxin, skin lesions 32
endotoxin, pertussis, cellular immune response 176
enrichment media, *Bordetellae* 317
enzymatic activities, AC toxin domain structure 263
enzymatic activities, pertussis toxin S1 subunit 44
epidemiological studies, serodiagnosis 325
epidemiology, pertussis 335
erythrocyte membrane, AC toxin treated 22
erythrocytes, human; AC toxin, hemolytic activity 27
Exotoxin A, diphtheria toxin, active-site residues 44
exposed contacts, IgA response 334
exposed contacts, serodiagnosis 326
F actin, microfilament disrupters 107
fatty acids
  heat-labile protein, smooth muscle contraction 36
  unsaturated, heat-labile toxin, inhibitory factors 34
FHA locus (*fhaB*) 217
FHA locus (*flaB*), adherence factors 91
filamentous hemagglutinin (FHA)
  acellular vaccines 312
  adherence 91, 100
  adhesion 131
  antibodies, patient 291
  antibody response, vaccine 305
  bacteria, attachment 99
  cholesterol 99
  CR3 95
  IgE response 179
  intracerebral challenge 148
  invasion 115
  lectin-like binding sites 103
  macrophages 94
  ORF, adherence factors 91

filamentous hemagglutinin (FHA) (cont'd)
  Salmonella typhimurium vaccines 189
  structure-function relationship 46
  T-cell response 166
  vaccine potency 40
fimbriae
  adherence 92
  B. pertussis strains 93
  intracerebral challenge 148
  Merieux vaccine 310
  production 251
  structure-function relationships 126
fluorescent antibody screening, pertussis 335
galactose, macrophages 94
gene autoregulation, S1 subunit 285
gene banks, porin gene 142
gene *bvgA* 230, 272
gene *bvgA*, transcription 222
gene *bvgB* 230
gene *bvgC* 230
gene *bvgS* 230, 272
gene cloning, porin 141
gene *cya* operon, regulation 236, 267
gene *cyaA* 248
gene *cyaB* 272
gene *cyaC* 264
gene *cyaC*, adenylate cyclase, hemolytic activity 267
gene *cyaD* 272
gene *cyaE* 247, 272
gene *fhaB*, transcription 222
gene *fim*, model for regulation 255
gene *fim*, promoter, location 253
gene *fim2* 253
gene *fim3* 253
gene *fimE* 256
gene *fimX* 251
gene *hlyC*, *cyaC* 272
gene structures
  filamentous hemagglutinin (FHA) 47
  porin 141
  S1 subunit 284
  vir locus 227
genetic studies, *vir* locus 225
guinea pigs, heat-labile toxin, skin lesions 30
heat-labile toxin, skin lesions, exogenous agents 30
HeLa 229 cells, invasion, *B. pertussis* 106, 115
HeLa cells 100
hemagglutination, pertussis toxin 74
hemolysis
  AC toxin 20, 233
  AC toxin, calcium and nickel 14
  AC toxin domain structure 263
  adenylate cyclase, hemolytic activity 235, 267
  calcium-independent hemolysins 47
  CyaA protein 235
hemorrhagic necrosis, heat-labile toxin 32
histology, disease process, mice 204
HIV patients, *B. pertussis* infections 124
HTE, serotonin and TCT binding studies 6
humoral response
  69-kDa protein, mice 82
  cellular immune response, correlation 177
  detoxified pertussis toxin 161
  pertussis toxin, IgE 184
  respiratory infections 197
  Salmonella typhimurium vaccines 189
  whole-cell and acellular vaccines 303
5-hydroxytryptamine
  TCT binding, respiratory epithelial cells 9
immune recognition, pertussis toxin, detoxified 38
immune response
  aerosol-infected mice 166
  detoxified pertussis toxin 161
  IgA 330
  pertussis infection 335
  porin, mice 145
immunity
  detoxified pertussis toxin 161
  murine aerosol infection model 166
  vaccines, immunological marker of protection 300
immunization, antibody response, mice 198
immunization schedule, DTP, United Kingdom 309
immunoblotting, pertussis toxin subunits 67
immunoblotting, serodiagnosis, pertussis 325
immunodominant regions, 69-kDa proteins 136
immunological characterization
  filamentous hemagglutinin (FHA) 48
  fimbriae 126
  G proteins, pertussis toxin 61
  pertussis toxin, S3 subunit 66
  pertussis toxin, B oligomer 66
  69-kDa proteins 136
infection, pertussis vaccines 300
infectious process
  fimbriae 126, 133
  immune response 331
  microbial gene expression 249

intracellular proliferation, *B. pertussis* 114
intracerebral challenge, 69-kDa protein 77, 86
intracerebral challenge, outer membrane vesicles 148
intramuscular injections, pertussis vaccines 302
intranasal challenge, 69-kDa protein 76
intranasal challenge, mice 127
invasion
  B. pertussis 115
  B. pertussis, HeLa 229 cells 106
  microfilament disrupters 110
ischemia, heat-labile toxin 30
isolation of *Bordetellae* 315, 336
isopropyl beta-D-thiogalactoside
  P*tac*-mediated gene expression 246
Japanese acellular vaccines 311
Jurkat cells 13
Kendrick test, P.69 278
laboratory methods, diagnosis, Swedish trial 291
lacZYA transcriptional fusions, construction 218
Lederle acellular vaccine 304
linoleic acid, heat-labile toxin, inhibitory factors 34
lipid A, heat-labile protein, hemorrhagic lesions 36
lipids, heat-labile toxin, skin lesions 32
lipooligosaccharide (endotoxin), serodiagnosis 324
lipopolysaccharide, ciliated cell-specific damage 10
lipopolysaccharide, T-cell and lymphokine 167
lymphocytosis (LPF), cellular immune response 176
lymphokines, aerosol-infected mice 167
lymphokines, IL-2/IL-4, aerosol-infected mice 167
macrophages
  B. pertussis 99
  human, adherence 91
  pulmonary alveolar, invasion 125
mapping techniques, 69-kDa proteins 136
mass spectrometry, 69-kDa protein 80
Merieux vaccine 304
Merieux vaccine, fimbrial antigens 310
$MgSO_4$, adenylate cyclase activity 271
mice, 69-kDa protein, model appropriateness 84
microagglutination, clinical trials 310
microfilament formation, invasion, *B. pertussis* 106
microspheres 201
mitogenicity, pertussis toxin B subunit 40
mitogenicity, PT-9K/129G 162
mod constitutive mutants 124
molecular biological studies
  69-kDa protein 79
  pertussis toxin, substrates 57
  porin 142
monoclonal antibodies
  1B7 38
  69-kDa proteins 136
  filamentous hemagglutinin (FHA) 48, 51
  fimbriae 100, 126
  G proteins 58
  Gt alpha 60
  macrophage surface proteins 94
  outer membrane vesicles 150
  pertactin 100
  pertactin RGD regions 106
mRNA, G_o alpha 58
mucosal immunity, respiratory infections 196
mutagenesis, G proteins, pertussis toxin 62
mutagenesis, in vitro, G proteins 58
mutational analysis, AC toxin 260
N-terminal region
  67-kDa protein 78
  69-kDa protein 76
  S1 subunit, autoregulation 285
NAD, pertussis toxin, S1 subunit 43
nasal IgA 330
nasal mucus, diagnosis, pertussis 334
nasal mucus, IgA, immune response 330
nasopharyngeal swabs, specimen collection 315
Netherlands WCV 154
NH_4Cl, Chinese hamster ovary cells 55
nickel, AC toxin 15
oleic acids, heat-labile toxin, inhibitory factors 34
oral immunization model, antibody formation 189
outer membrane proteins,
  intracerebral challenge 148
outer membrane vesicle, intracerebral challenge 148
outer membrane vesicle, preparation 149
P. mirabilis, hemolysin; filamentous hemagglutinin 47
P388.D1 cells 100
pathogenic mechanisms, transposon insertion 243
patients (*B. pertussis*)
  age, risk groups 340
  antibody response 338
  asymptomatic exposed contacts 175
  cellular immune response 175
  nasal IgA 330
  serodiagnosis 324
  specimen collection 315
Pepscan, epitopes mapping 138
peptides, synthetic
  pertactin 137
  pertussis toxin, S3 subunit 66

peptides, synthetic (cont'd)
  S1 subunit 163
peptidoglycan, TCT biosynthesis 7
pertactin
  adherence 92, 100
  immunodominant regions 136
  invasion, *B. pertussis* 106
  RGD sequence 111
pertussis toxin
  antibodies, patient 291
  antibody response, vaccine 305
  assembly 46
  ciliated cells 94
  detoxified 37
  detoxified, cell mediated immunity 174
  disease process 202
  eukaryotic cells, entry 53
  genetically detoxified 161
  IgE response 179, 184
  mutants, PT-9K/129G 31, 161
  nasopharyngeal swabs 318
  operon (*ptx*) 37, 92, 217
  PT-9K/129G, detoxified 37, 161
  rabbits, response 259
  structure-function relationships 41
  substrates 57
  subunits, S-2 and S-3 52
  T-cell response 166
  toxoided, intracerebral challenge 148
  vaccine efficacy 297
pertussis toxin operon (*ptx*) 37, 92, 217
pertussis toxin, S1 subunit 37, 40
  ADP-ribosyltransferase activity 42
  gene autoregulation 281
  holotoxin assembly 46
  immunity 163
  L-site 43
  NAD 43
pertussis toxin, S2 subunit 71
  galactose recognition site 94
pertussis toxin, S3 subunit 66
  sialic acid recognition site 94
pertussis toxoid, acellular vaccines 312
phase variation, *fim* genes 251
phase variation, fimbriae 253
phenotypic modulation, *cya* operon 267
Philadelphia, PA, pertussis epidemiology 335
plasma membrane, pertussis toxin, entry 55
plasmids, pLAFRII 46
plasmids, *Salmonella typhimurium* 189
polymyxin 124
polymyxin B, *B. pertussis* sensitivity 117
population risk groups, pertussis 340
porin, outer membrane proteins 141, 156
proline, SDS-PAGE 278
protein, 67-kDa 78
protein, 68-kDa, *B. bronchiseptica* 138
protein, 69-kDa 75
  adherence 92, 100
  cellular immune response 176
  expression and purification 275
  immunodominant regions 136
  invasion 106, 115
  recombinant P.69, expression and purification 275
  T-cell response 166
  vaccine potency 40
protein, 70-kDa, *B. parapertussis* 138
protein, BvgB 230
protein, BvgC 230
protein, BvgS 230
protein, CyaA
  hemolytic moiety, function 240
  secretion 271
  secretion and activity 234
protein, Fim 2 126
protein, Fim 3 126
protein, G
  filamentous hemagglutinin (FHA) 51
  pertussis toxin 54, 57
  pertussis toxin, S1 subunit 43
protein, G_o alpha, pertussis toxin 57
protein, G_t alpha, pertussis toxin 57
protein, outer membrane; porin 141
ptxA::lacZYA fusion, *vir* locus 223
pulmonary pathology 99
purification, porin 142
repressors, *cya* operon 271
respiratory challenge, lethal, 69-kDa protein 83
respiratory epithelial cells, TCT toxicity 8
respiratory infections
  fimbriae, mice 131
  mucosal immunity 196
  T-cell response 166
respiratory response, fimbrial antigens 331
RGD sequence, filamentous hemagglutinin 95
rhodopsin, pertussis toxin, G proteins 60
Salmonella typhimurium oral vaccine
  B. pertussis antibodies 189

SDS gel electrophoresis, 69-kDa protein 83
SDS-PAGE, P.69 and 69-kDa protein, size 278
secretory IgA 330
sequence analysis, pertactin 136
serodiagnosis, pertussis 322
serological assays, vaccine efficacy 294
serologies 337
serotonin, TCT binding studies 6
shuttle transposon mutagenesis 272
signal transduction, *vir* system 230
site selection, clinical trials 311
skin homogenates
    heat-labile toxin, inhibitory factors 34
skin lesions, heat-labile toxin, exogenous agents 30
smooth muscle contraction
    heat-labile protein, fatty acids 36
specimen collection and transport, *B. pertussis* 315
standards, sera 309
structure-function relationships
    AC toxin 15, 27
    filamentous hemagglutinin (FHA) 46
    fimbriae 126
    pertussis toxin 41
    synthetic peptides probes 71
study design, clinical trials 312
subclinical infections, pertussis 341
subcutaneous injections, pertussis vaccines 302
substrates, pertussis toxin 57
surface proteins, bacterial adherence 100
surface proteins, invasion process 110
T-helper cells, human 13
T lymphocytes
    activation assays 172
    aerosol-infected mice 166
    S1 subunit 163
TCT 5
TCT, analogs 6
TCT, structure-function relationships 8
temperature, *vir*-regulation 232
toxin activity, AC toxin domain structure 263
*tox*R, *vir* locus 232
transcription, *fhaB* and *bvg* activation 222
transcription, S1 subunit truncation, PT 284
transcriptional fusions, *bvg* regulation 218
transport, AC toxin domain structure 263
transposons
    insertion mutations, pathogenic mechanisms 243
    mutagenesis, shuttle 272
    Tn*5tac*1, insertion mutations 245
U937 cells, invasion, *B. pertussis* 115
UK adsorbed whole-cell DTP vaccine 308
United Kingdom, clinical trials 303
United States government-sponsored trial 311
vaccination schedule 310
    clinical trials 313
vaccine potence, *in vitro* cell adhesion inhibition assay 133
vaccines, acellular pertussis 40, 311
    AC toxin 205
    adenylate cyclase-hemolysin 209
    antibody response 303
    clinical trials, choice of antigen 312
    IgE 179
    IgE response 184
    Japanese acellular vaccines 311
    Lederle 304
    P. 69-kDa protein 75, 278
    protection over time 300, 310
    PT-9K/129G 161
    safety 48
    T lymphocyte response 172
    virulence factors 202
vaccines, DTP 303, 310
vaccines, efficacy 299, 313
    2-component acellular pertussis vaccine 297
    acellular pertussis vaccines 311
    laboratory conformation of infections 291
    serological assays 294
    single-component acellular pertussis vaccine 297
vaccines, pertussis
    aluminum adjuvants 187
    B-cell-mediated immunity 199
    bacterial colonization and infection 300
    case definitions 301
    components 299
    costs 313
    detoxified pertussis toxin 37
    immunological marker of protection 300
    JNIH-6, Swedish efficacy trial 293, 295, 299
    JNIH-7 292
    JNIH-7; Swedish efficacy trial 292, 295, 299
    number of doses 301
    outer membrane vesicles 152
    route 302
    synthetic 71
vaccines, safety
    69-kDa protein 81
    acellular pertussis vaccine 311

vaccines, safety (cont'd)
  PT-9K/129G 162
  whole-cell and acellular vaccines 48, 306
vaccines, whole-cell pertussis 40
  antibody response 303
  IgE response 186
  nasal IgA 330
  protection over time 300
vaccines
  antifimbrial antibodies 133
  Dutch DPTP 154
  oral 193
  recent research, overview ix
  Salmonella typhimurium 189
  UK adsorbed whole-cell DTP 308
Vero cells, adhesion 130
vir locus 217
  cya operon, regulatory sites 267
  cya promoter 239
  fhaB and *bvgA* transcription 222
  fim genes 251
  genetic studies 225
  invasion 122
  transcriptional fusions 218
vir regulated proteins 135
vir regulation, adenylate cyclase 114
Vir$^-$ mutants, invasion, *B. pertussis* 115
Vir$^-$ mutants, respiratory infections 132
virulence factors 260, 275
  AC toxin 13, 20
  adenylate cyclase 207
  adenylate cyclase and hemolysin 233
  adenylate cyclase, regulation 272
  disease process 202
  genetic studies 225
  mutants, mixed infections 203
  regulation and transcription 217
  S1 subunit, pertussis toxin 281
  structure function relationships 41
  TCT 5
  vir-encoded proteins, regulation 226
volunteers, children, Swedish efficacy trial 291, 295
volunteers, children, United Kingdom 303
whoops, vaccine efficacy, clinical criteria 295
X-mode *B. pertussis*, vaccines 154

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